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AMENDMENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	H. Lowenheim	Attorney Docket No.:	SOPH116953
Application No.:	09/622,719	Group Art Unit:	1635
Filed:	October 18, 2000	Examiner:	K.A. Lacourciere

Title: METHOD FOR THE TREATMENT OF DISEASES OR DISORDERS OF THE INNER EAR

DECLARATION Of JONATHAN KIL

Seattle, Washington 98101

March 5, 2003

TO THE COMMISSIONER FOR PATENTS:

I, Dr. Jonathan Kil, declare as follows:

1. I am the CEO of Sound Pharmaceuticals, Inc., Seattle, Washington, and I am familiar with the subject matter disclosed and claimed in the above-identified application.

2. A copy of my *curriculum vitae* is appended hereto as Attachment B.

3. I have considered the Office Action dated September 10, 2002, issued in the above-identified application. It is my understanding that the Examiner has rejected claims in the application on the basis of lack of enablement. The Examiner has relied on Pfister & Lowenheim (2002) *Gentherapeutische Aspecte am Innohr*, pp. 50-7, Lowenheim et al. (1999) *Proc. Natl. Acad. Sci. U.S.A.* 96:4084-8, and Chen & Segil (1999) *Development* 126:1581-90 to conclude that it would take undue experimentation to control the development of sensory cells by administering an inhibitor of cell cycle inhibitors. In addition, the Examiner has relied on Agrawal & Kandimalla (2000) *Molec. Med. Today* 6:72-81, Branch (1998) *TIBS* 23:45-50, Green et al. (2000) *J. Am. Coll. Surg.* 191:93-105, and Jen & Gerwirtz (2000) *Stem Cells* 18:307-319 to conclude that the therapeutic use of antisense oligonucleotides was an unpredictable art at the time the invention was made.

Considered KAL 05-30-03

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4. My colleagues and I conducted the following experiments to evaluate the effect of $p27^{Kip1}$ antisense oligonucleotide SPI5101 on proliferation on supporting cells in the organ of Corti of the inner ear.

5. To test whether inhibition of the $p27^{Kip1}$ gene product would allow post-mitotic, terminally differentiated $p27^{Kip1+/+}$ supporting cells to proliferate, we treated wild-type cochlear cultures with a 15mer $p27^{Kip1}$ antisense oligonucleotide (SPI5101), that had been developed to inhibit human and murine p27Kip1 (Coats et al. (1996) *Science* 272(5263):877-80). Cultures were treated with 1 mM neomycin sulfate, an ototoxic antibiotic, for 48 hours. SPI5101 was then transfected to the remaining supporting cells by lipofection for 24-48 hours at 40nM in culture media containing 1 micromolar bromodeoxyuridine (BrdU). Antisense oligonucleotides that were FITC-conjugated could be detected within live supporting cells after 24 hours of lipofection. After 48 hours of lipofection, 18.6 ± 6.0 FITC-positive supporting cells were observed per 500 micrometer length of sensory epithelium. Cultures treated with SPI5101 possessed an average of 5.6 ± 3.0 BrdU labeled supporting cells per 500 micrometers ($P < 0.001$), while cultures treated with lipid only contained 0.3 ± 0.5, and cultures treated with random sense oligonucleotides had an average of 0.5 ± 0.8 BrdU labeled cells. BrdU-positive supporting cells were observed in all cochlear cultures treated with SPI5101.

6. To validate $p27^{Kip1}$ antisense antagonism as a viable therapeutic modality for the induction of proliferation in the cochlea *in vivo*, 10 micromolar SPI5101 was delivered to adult Guinea pigs. SPI5101 drug delivery (0.5 microliter/hr, 7 days) was by mini-osmotic pump through a catheter to the inner ear of Guinea pigs previously exposed to an ototoxic lesion (gentamycin 0.125 mg/ml, 0.5 microliter/hr, 7 days). In addition to SPI5101, BrdU was added to allow identification of proliferating cells. Western blot analysis from perfused cochlea showed decreases in p27 protein levels relative to control cochlea following SPI5101 delivery (see

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FIGURE 1). Two out of five animals in this series treated with 10 micromolar SPI5101 showed proliferation in the organ of Corti as observed in both whole mount and cross-sectioned tissues. In contrast, control animals receiving BrdU alone or in combination with a GC-content-matched random sense control oligonucleotide did not show proliferation in the organ of Corti.

7. My colleagues and I have identified novel antisense $p27^{Kip1}$ oligonucleotides that are significantly better at reducing both $p27^{Kip1}$ mRNA and protein levels than SPI5101 by evaluating variations in chemical composition of antisense $p27^{Kip1}$ oligonucleotides (*i.e.*, phosphate backbone linkage), length (15-25mer), and sequence. To date, the most significant improvement has come through sequence and length variation of the oligonucleotide. A 25mer phosphothioate (SPI5505) emerged as the best candidate for decreasing both cellular $p27^{Kip1}$ RNA and protein levels, as described below.

8. The effect of antisense oligonucleotides on $p27^{Kip1}$ mRNA and protein levels was evaluated by lipofecting the following oligonucleotides into NIH3T3 cells at 100nM in three separate culture plates: SPI5101, a random sense control for SPI5101 (SPI5102), a sequence optimized phosphothioate 25 base antisense oligonucleotide (SPI5505), and a random sense control for SPI5505 (SPI5514). Real time quantitative PCR (TaqMan) was used to determine $p27^{Kip1}$ mRNA levels. Densitometric analysis of Western blots was used to determine $p27^{Kip1}$ protein levels, which were standardized relative to alpha-tubulin levels in each sample. A significant reduction in $p27^{Kip1}$ mRNA levels was observed using SPI5505 compared to SPI5101 (FIGURE 2). Moreover, SPI5505 results in a dramatic dose-dependant reduction in $p27^{Kip1}$ mRNA levels in lipofected NIH3T3 cells as compared to the sense control (see FIGURE 3). Similarly, SPI5505 results in a dramatic dose-dependent reduction in $p27^{Kip1}$ protein levels in serum starved NIH3T3 cells as compared to the sense control (see FIGURE 4).

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9. All statements made herein and of my own knowledge are true, and all statements made on information and belief are believed to be true; and further, these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful, false statements may jeopardize the validity of the above-identified application or any patent issued thereon.

Respectfully submitted,

Jonathan Kil, M.D.

Date: March 5, 2007

:KBB

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JONATHAN KIL, M.D.

Education:

University of Virginia	M.D.	1992-1996
Georgetown University	MD/PhD (candidate)	1990-1992
University of California, Irvine	B.S. in Biological Sciences	1985-1989

Experience:

2001-	Founder, President, CEO Sound Pharmaceuticals Inc., Seattle, WA
1998-2001	Founder, President, CEO, CSO Otogene USA, Inc., Seattle, WA Member of the Vorstand, Otogene AG, Tuebingen, Germany
1996-1998	Senior Fellow Department of Otolaryngology-HNS and VM Bloedel HRC University of Washington
1992-1996	M.D./Ph.D. (candidate) Departments of Neurosciences and Otolaryngology-HNS University of Virginia (UVA)
1990-1992	M.D./Ph.D. candidate (transferred to UVA to conduct inner ear research) Departments of Cell Biology and Otolaryngology-HNS Georgetown University
1989-1990	Research Assistant Department of Anatomy and Neurobiology University of California, Irvine

Grants:

2000-2002	NIH SBIR Phase II #DC04258-02, P.I.
1999-2000	NIH STTR Phase I #DC04258-01, P.I.
1996-1998	Individual NRSA Postdoctoral Research Fellowship #DC00247, P.I.
1991-1992	American Hearing Research Foundation Research Grant, Co-investigator

Awards/Honors:

1995	Association for Research in Otolaryngology Medical Student Travel Award
1995	Winn Medical Student Scholarship for Otolaryngology-HNS
1991	Achievement Reward for College Scientists (ARCS) Foundation Scholarship
1989	Ralph W. Gerard Award for Outstanding Research
1989	Excellence in Research Honors

Publications:

Kil, J., R. Gu, A. H. Sudra, E. D. Lynch, T. Hasson, H. Löwenheim, E. Negrou§, M. L. Fero. (2002) $p27^{Kip1}$ maintains terminal differentiation in the organ of Corti. (submitted)

Kopke, R.D, Coleman, J.K.M., Huang, X., Weisskopf, P.A., Jackson, R.L., Liu, J., Hoffer, M.E., Wood, K., Kil, J., Van De Water, T.R. Novel Strategies to Prevent and Reverse Noise-Induced

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Morris, M.S., J. Kil, and M.J. Carvlin. 1993. Magnetic Resonance Imaging of Perilymphatic Fistula. *Laryngoscope*. 103:729-733.

Kil, J. 1989. Developmental plasticity in the gerbil auditory brainstem. J. Undergraduate Research in the Biological Sciences, Univ.of California, Irvine. 19:409-419.

Meetings (selected abstracts):

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Kil, J., Hanigan, M. H., Taylor, Jr., P.T. and Hashisaki, G.T. (1996) Localization of gamma-glutamyl transpeptidase in the chick inner ear sensory epithelia. Soc. Neuro. Abs. 22, 1622.

Memberships:

Association for Research in Otolaryngology
Society for Neuroscience
IBRO
AAAS

ATTACHMENT C

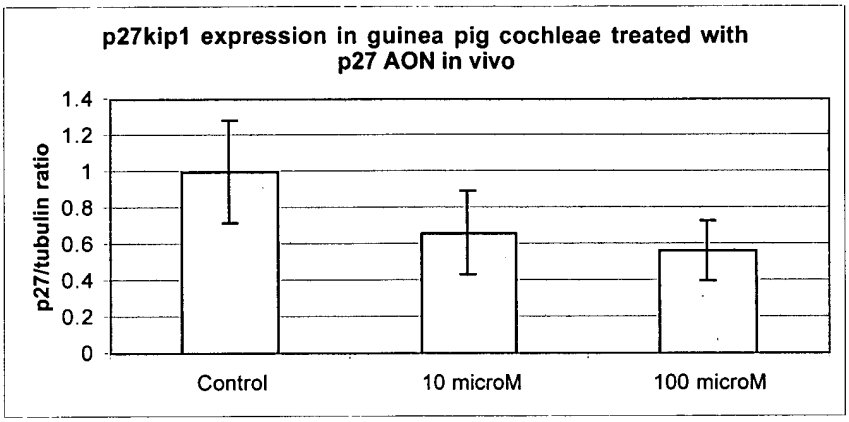

FIGURE 1

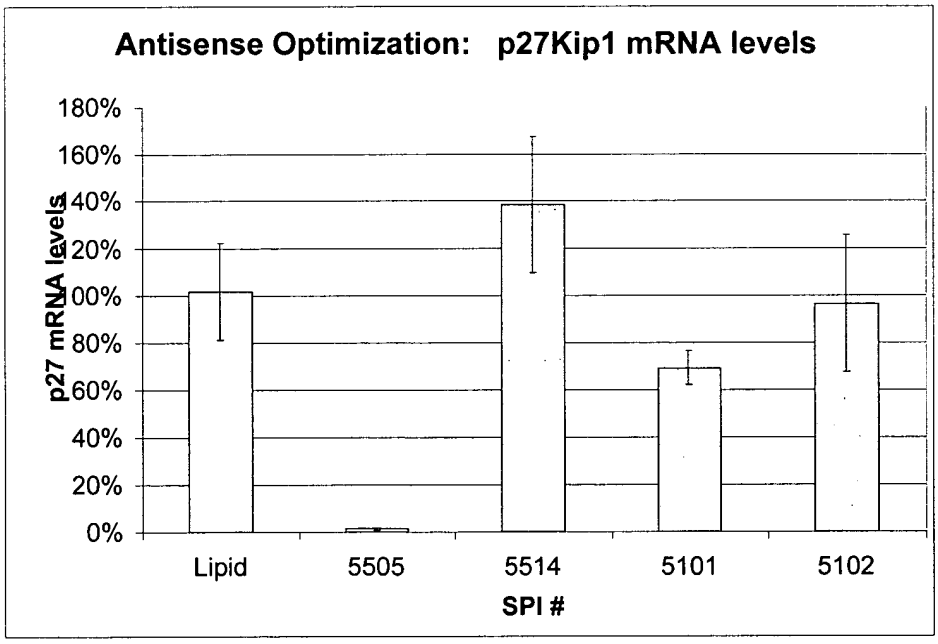

FIGURE 2

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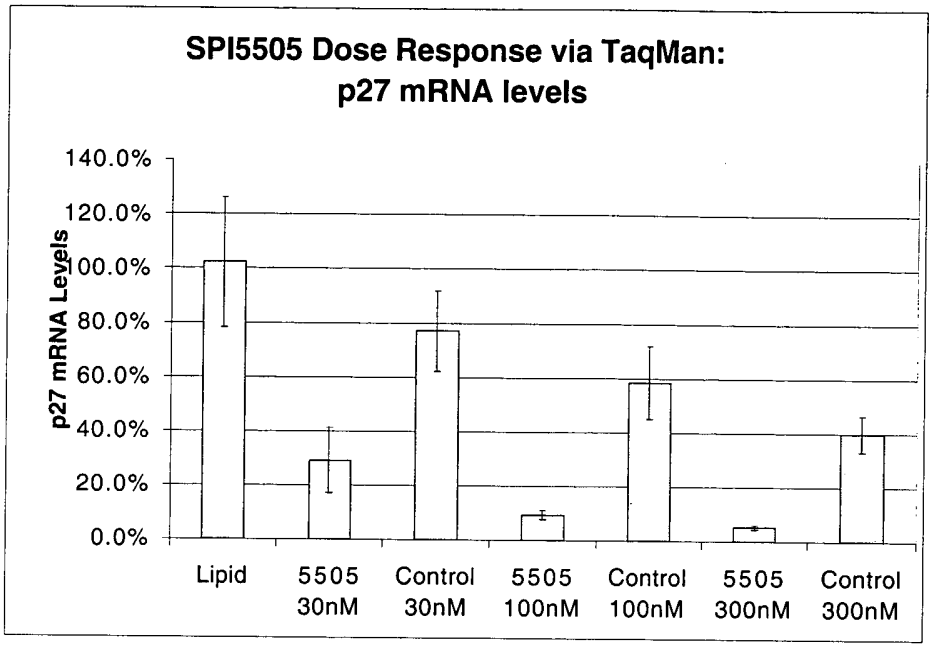

FIGURE 3

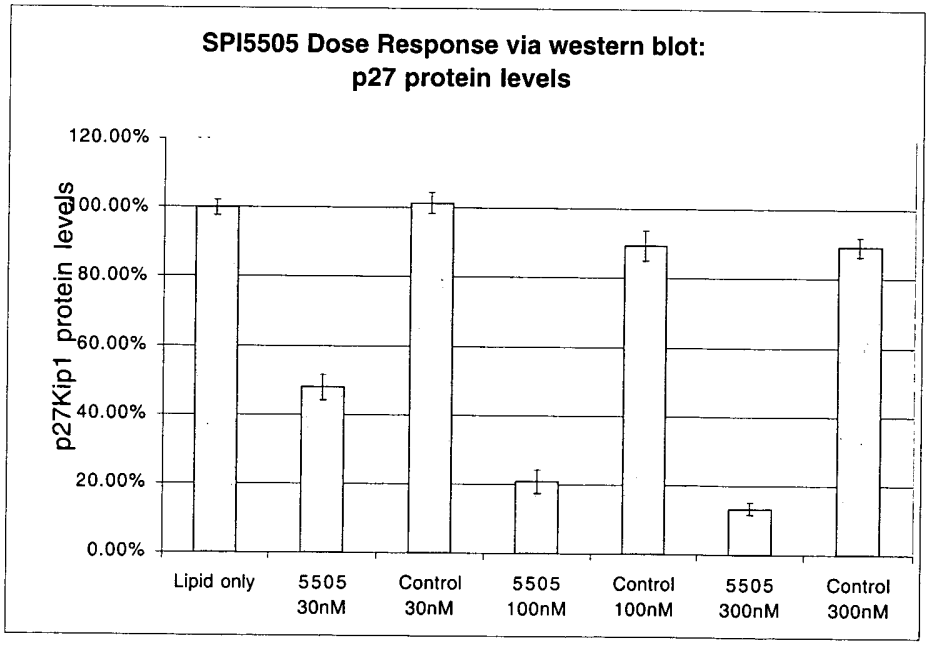

FIGURE 4

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1: Brain Res Mol Brain Res 1998 Mar 30;55(1):151-64 Related Articles, Links

ELSEVIER SCIENCE
FULL-TEXT ARTICLE

Antisense oligonucleotides to the GluR2 AMPA receptor subunit modify excitatory synaptic transmission in vivo.

d'Aldin C, Caicedo A, Ruel J, Renard N, Pujol R, Puel JL.

INSERM U.254 Universite Montpellier I, Laboratoire de Neurobiologie de l'Audition, Plasticite Synaptique, CHR Saint Charles, Montpellier, France.

In the brain, fast wxcitatory synaptic transmission is mostly mediated by the alpha-amino-3-hydroxy-5-methyl-isoxazole-propionic acid (AMPA) subtype of the glutamate receptors. Molecular cloning has revealed that four subunits, GluR1, GluR2, GluR3, and GluR4 form heteromeric receptors with high affinity for AMPA. Because antagonists and agonists do not discriminate between individual AMPA receptor subunits, we decided to use antisense oligonucleotides to block the expression of the GluR2 subunit within the receptor complex in adult animals. In the present study, we exploited several advantages afforded by the guinea pig cochlea to determine whether an antisense oligonucleotide directed to the mRNA of the GluR2 subunit could modify primary auditory neurotransmission. While a random probe with the same base composition had no effect, a GluR2 antisense oligonucleotide, continuously delivered into the cochlea, transiently reduced the compound action potential and diminished spontaneous activity of single auditory nerve fibers. Although antisense oligonucleotides penetrated a variety of cells, their effect could be physiologically localized to a single site of GluR2 antisense probe action, the primary auditory neuron. Subunit specificity of this effect was confirmed by a significant reduction in GluR2/3, but not GluR4 immunoreactivity in primary auditory neurons. Besides being the first demonstration that transient knockout of GluR2 subunit in adult animal modifies excitatory synaptic transmission in vivo, these results support the use of the antisense strategy as a powerful tool for blocking expression of any gene in the cochlea.

PMID: 9645970 [PubMed - indexed for MEDLINE]

1: Hear Res 1999 Sep;135(1-2):105-12 Related Articles, Links

Phosphorothioate oligodeoxynucleotides can selectively alter neuronal activity in the cochlea.

LeBlanc CS, Fallon M, Parker MS, Skellett R, Bobbin RP.

Department of Otorhinolaryngology and Biocommunication, Louisiana State University Medical Center, New Orleans 70112-2234, USA.

A growing body of evidence indicates that extracellular adenosine triphosphate (ATP) may have a major role in cochlear function. Antagonists of ionotropic ATP receptors (P2X2) have significant effects on cochlear potentials and distortion product otoacoustic emissions (DPOAEs). We tested whether antisense oligodeoxynucleotides (ODNs) would mimic the functional deficiencies induced by the ATP antagonists through binding to P2X2 ATP receptor mRNA and thereby reduce the number of ATP receptors expressed in the membrane of the cells. Both a phosphorothioate ODN (S-ODN) antisense and a phosphodiester ODN (P-ODN) antisense to the P2X2 sequence and random sense ODNs containing 21 nucleotides were administered chronically (7 days) to the guinea pig cochlea via the perilymph compartment. Sound evoked cochlear potentials (cochlear microphonic; summating potential; compound action potential of the auditory nerve, CAP; latency of the first negative peak in the CAP, N1 latency) and DPOAEs were monitored to assess the effects of the ODNs. Results indicate that the phosphorothioate derivatives of both the antisense and random sense ODNs suppressed the CAP and prolonged the N1 latency with no significant effect on the other parameters. The P-ODNs had no effect. Since both the antisense and random sense S-ODNs had the same effect, we conclude that the S-ODNs affected neuronal function in a manner that did not involve binding to the ATP receptor mRNA.

PMID: 10491959 [PubMed - indexed for MEDLINE]

ELSEVIER

Hearing Research 128 (1999) 61–69

HEARING RESEARCH

Cationic liposome mediated transgene expression in the guinea pig cochlea

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Received 25 June 1998; received in revised form 1 October 1998; accepted 12 October 1998

Abstract

Sensorineural hearing loss affects nearly 10% of the American population that is refractory to conventional therapy. Gene therapy represents an intervention with potential therapeutic efficacy. We studied the feasibility of cationic liposome mediated gene transfer within the guinea pig cochlea in vivo following direct microinjection into the cochlea. Transgene expression was persistent up to 14 days in the neurosensory epithelia and surrounding tissue without toxicity and inflammation in the target organ. This study represents the first successful use of cationic liposomes for cochlear gene transfer thus providing a safe and rapid alternative to the use of recombinant viral vectors in gene therapy for inner ear disorders.

Key words: Mammalian cochlea; Cationic liposome; Gene therapy; Transgene expression

1. Introduction

Studies investigating cochlear gene therapy have been initiated using a variety of viral vectors and guinea pig as the animal model. Several laboratories have established the relative feasibility of in vivo introduction and expression of a marker gene within the cochlea of a guinea pig, using viral vectors. These include the replication-defective derivative of the adeno-associated virus (AAV) (Lalwani et al., 1996) and the adenovirus (Ad) (Raphael et al., 1996). The use of these vectors in cochlear gene therapy studies has also revealed their major drawbacks. Relatively low viral titers and their prolonged preparation time represent the major technical limitation with the use of AAV, while the use of Ad is beset with problems associated with its immunogenicity. Safety concerns and the difficulty with readily obtaining high concentrations of recombinant virus may be circumvented with the use of cationic lipid vesicles or liposomes as gene transfer vectors (Lee and Huang, 1997; Flotte and Ferkol, 1997; Gao and Huang, 1995).

Cationic liposomes are non-immunogenic and easily prepared in large amounts. The liposomes can then be mixed with DNA of virtually any size to yield complexes that are held together through ionic interactions. Although the complexes are not stable structurally in the presence of serum, binding of the liposome-DNA complex, or lipoplex, to the plasma membrane results in transfection of many cell types (Felgner, 1997; Stewart et al., 1992). The lipoplex does not replicate or recombine and in general does not integrate into the genome of the host cell. Hence, the cationic liposome bound DNA poses minimal risk of insertional mutagenesis.

The safety and effectiveness of the cationic liposomes for gene delivery has been demonstrated in vitro and in vivo (local injection) in many studies including two clinical trials. In vivo studies have shown the use of liposome mediated gene delivery for tumor control and suppression resulting in longer survival in mouse model systems (Hsiao et al., 1997). Liposome mediated gene transfer has been clinically applied in the treatment of

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cystic fibrosis. Direct administration of liposome-DNA (CFTR cDNA) complexes to the nasal epithelium of patients with cystic fibrosis yielded functional gene transfer in six of eight patients, with two patients showing transient functional correction of the CF chloride transport abnormality. The treated patients did not display acute adverse effects (Gill et al., 1997).

The use of liposomes as gene transfer vectors has also revealed several potential limitations. Their relative absence of tissue specificity represents one such limitation. Neutral or negatively charged liposomes have been found to be taken up by macrophages after systemic injection (Allen, 1994). This shortcoming has been resolved by incorporating targeting ligands, including transferrin (Stavridis et al., 1986) and surfactant proteins (Walther et al., 1993) in the liposome-DNA complex, thus facilitating greater cell/tissue specificity during gene delivery. Compared with viral vectors, liposome-mediated gene delivery has been inefficient. However, modification of the liposome formulation, including changes in neutral lipids, has been shown to have profound effects of efficiency of transfection (Hong et al., 1997). Thus, transfection efficiency of liposome-DNA complexes in some tissues has approached that of recombinant viruses. Inefficient DNA entry into cytoplasm and translocation to the nucleus are also considered to hinder expression of liposome complexed genes. This factor is being addressed through inclusion of viral components within the liposomes to enhance their nuclear entry and survival of the conjugated DNA (Kaneda et al., 1989). A related concern with the use of liposomes is the duration of transgene expression. Expression of most transgenes delivered by cationic liposomes has been shown to last for a week or less. However, some studies have shown transgene expression to last several weeks; both six and nine weeks duration of expression have been documented using liposome mediated gene transfer (Jiao et al., 1992; Nabel et al., 1990; Zhu et al., 1993).

The shortcomings associated with the use of cationic liposomes are significantly counterbalanced by a number of critical attributes. These include their ability to use a range of DNA constructs – from simple plasmids to chromosomal fragments, their ease of preparation in large concentrations, relatively simple transfection protocols and their non-immunogenicity and safety. This study reports the use of cationic liposomes to introduce an exogenous marker gene within the guinea pig cochlea.

2. Materials and methods

2.1. Preparation of liposomes

Cationic liposomes were prepared in 5% (w/v) dextrose solution as previously described (Hong et al., 1997). Briefly, DDAB was mixed with cholesterol in chloroform at 1:1 molar ratio. The mixture was evaporated and hydrated in 5% dextrose solution. The suspension was then sonicated and stored under argon at 4°C.

2.2. Preparation of transfection complexes

Transfection complexes were formed by pipetting plasmid into a liposome suspension of equal volume. DNA (1 μg): dimethyldioctadecylammonium bromide (DDAB)-cholesterol (12 nmol). The final DNA concentration of the complexes was 200 μg/ml.

2.3. Animal model

The Hartley guinea pig was used as the animal model due to the relatively large size of the cochlea compared to mouse and rat, and ease of the surgical manipulation in this species.

2.4. Number of animals

A total of five animals were infused and assessed for individual time periods. Four additional animals were infused with liposomes alone and served as negative controls.

2.5. Animal surgery and local delivery of the liposome-DNA complex

Two methods of gene vehicle delivery were employed. Of these two, the direct injection method was the primary mode of gene delivery. Animals were initially anesthetized with a combination of intramuscular ketamine (50 mg/kg) and the analgesic xylazine (9 mg/kg), and administered antibiotic prophylaxis using 4 mg of trimethoprim and 20 mg of sulfadiazine.

Direct injection involved a post-auricular incision, a wide opening of the bulla and visualization of the round window membrane after minimal bone removal in that area. Using a micro-manipulator and a microsyringe (Unimetrics) armed with a glass needle, the round window membrane was punctured. Ten μl of mixture was injected over a period of 20 min. Total operating time was in the order of 30–40 min.

In the osmotic minipump infusion method the tympanic bulla was exposed via a post-auricular incision and opened to allow visualization of the basal turn of the cochlea. A cochleostomy about 1 mm inferior to the round window was fashioned using a 0.5-mm diamond burr. The basilar membrane, visible through the cochleostomy, allows confirmation of the correct positioning of the cochleostomy. The catheter of the pump was then introduced into the basal turn of the cochlea via

the cochleostomy. The body of the pump was inserted in a subcutaneous pocket. The skin incision was closed in layers. This method required less operating time, in the order of 15–20 min. Prior to surgery, Alzet osmotic minipumps model 1007 (Alza Corporation, Palo Alto, CA, USA) were prepared by connecting PE50 and PE10 polyethylene tubing (Intramedic, Becton Dickinson, Parsippany, NJ, USA) to the flow moderator on the pump. The flow rate of 1007 Alzet minipump is 0.5 μl/h and delivers its entire reservoir of 100 μl over 8.3 days. The pumps were primed by filling with the relevant infusate, under sterile conditions, and incubating overnight at 37°C.

2.6. Tissue processing

At the time of sacrifice, the animals were perfusion-fixed with a solution of 4.0% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Animals were sacrificed at various time intervals with an intraperitoneal overdose of sodium pentobarbital (250 mg/kg) and bilateral thoracotomy. Temporal bones were harvested from both sides of the head. Each bulla was opened using rongeurs to expose the cochlea. The stapes was removed and the cochlea fixed by perfusion of 4.0% paraformaldehyde through the round window. The cochlea was then removed from the remaining temporal bone and immersed in 4.0% paraformaldehyde overnight at 4°C. After complete fixation, specimens were decalcified in 0.2 M EDTA/1× PBS/4.0% paraformaldehyde for 2–3 weeks with at least three solution changes. Following decalcification, the specimens were placed in 0.9% saline, dehydrated through a graded alcohol series and then equilibrated in xylenes. Specimens are embedded in paraffin and sectioned at 6–8 μm on a microtome (Leica RM2035).

2.7. Immunohistochemistry for detection of β-gal expression and cell-mediated immune response

The paraffin-embedded cochlear sections were dewaxed, blocked with 10% NHS, 0.1% Tween 20 in PBS and then hybridized overnight with the relevant primary antibody.

β-gal expression was detected using mouse anti-*E. coli* β-gal antibody. The sections were washed to remove unbound antibody and then hybridized with a secondary antibody (biotin labeled anti-mouse IgG monoclonal antibody). The bound label was amplified using the ABC reagent (Vector) and then developed with DAB. Sections were examined under low and high power magnification and the presence or absence of staining in different parts and their relative intensity noted.

Presence of cell-mediated immune response was characterized using antibodies against the guinea pig T4 lymphocytes (anti-CD45 antibody, clone no. 1H-1) and macrophages (anti-L1 antibodies, clone no. MAC387). Both of these antibodies are supplied by Serotec, Raleigh, NC, USA. The hybridized sections were then processed as described above.

2.8. Detection of the transgene within tissue sections via PCR analysis

DNA extracted from tissue sections was characterized for the presence of the liposome-β-gal complex via PCR analysis. Briefly, paraffin embedded tissue sections were scraped off the slides and incubated at 94°C for 20 min in the presence of 50 μl of 1% Triton X-100. One μl of the extract was then used as a template along with 10 picomoles of forward and reverse β-gal specific primers (β-galA: CGG GTT GTT ACT CGC TCA CAT TTA, and β-galB: CGC ACG ATA GAG ATT CGG GAT TTC) in a 20-μl PCR reaction with standard concentrations of other reagents. The conditions of PCR amplification were as follows: 94°C for 4 min; then, 5 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 1 min; then 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min followed by an extension for 5 min at 72°C. Appearance of a 450-bp long PCR product resolved on a 1% agarose gel is indicative of the presence of the transgene within tissue.

3. Results

3.1. Immunohistochemical characterization of the transgene expression

Tissue sections from guinea pig cochlea injected with liposome-β-gal and harvested at 1, 3, 7 and 14 days were assayed in situ for expression of the transgene product via immunohistochemistry. Immunoreactivity to polyclonal β-gal antibodies, detected through DAB staining, was observed in the β-gal-injected cochleae harvested at 3, 7 (Fig. 1A) and 14 days (Fig. 1B and C). Nearly all tissue types within the liposome injected cochleae – surrounding the cochlear duct as well as within the bony modiolus and extending from the base to the apex – were positive for β-gal expression. The transfected tissue types include the spiral ligament, spiral limbus, organ of Corti, Reissner's membrane and the spiral ganglia containing the primary auditory neurons. A tissue type that did not display β-gal immunoreactivity was the stria vascularis.

Amongst the transfected tissues, the spiral ligament expressed most intense β-gal immunoreactivity. Organ of Corti, the auditory sensory neuroepithelia, is also transfected as illustrated in Fig. 1C. The three rows of outer hair cells (OHC) and the supporting cells that surround these sensory cells are clearly visible in this

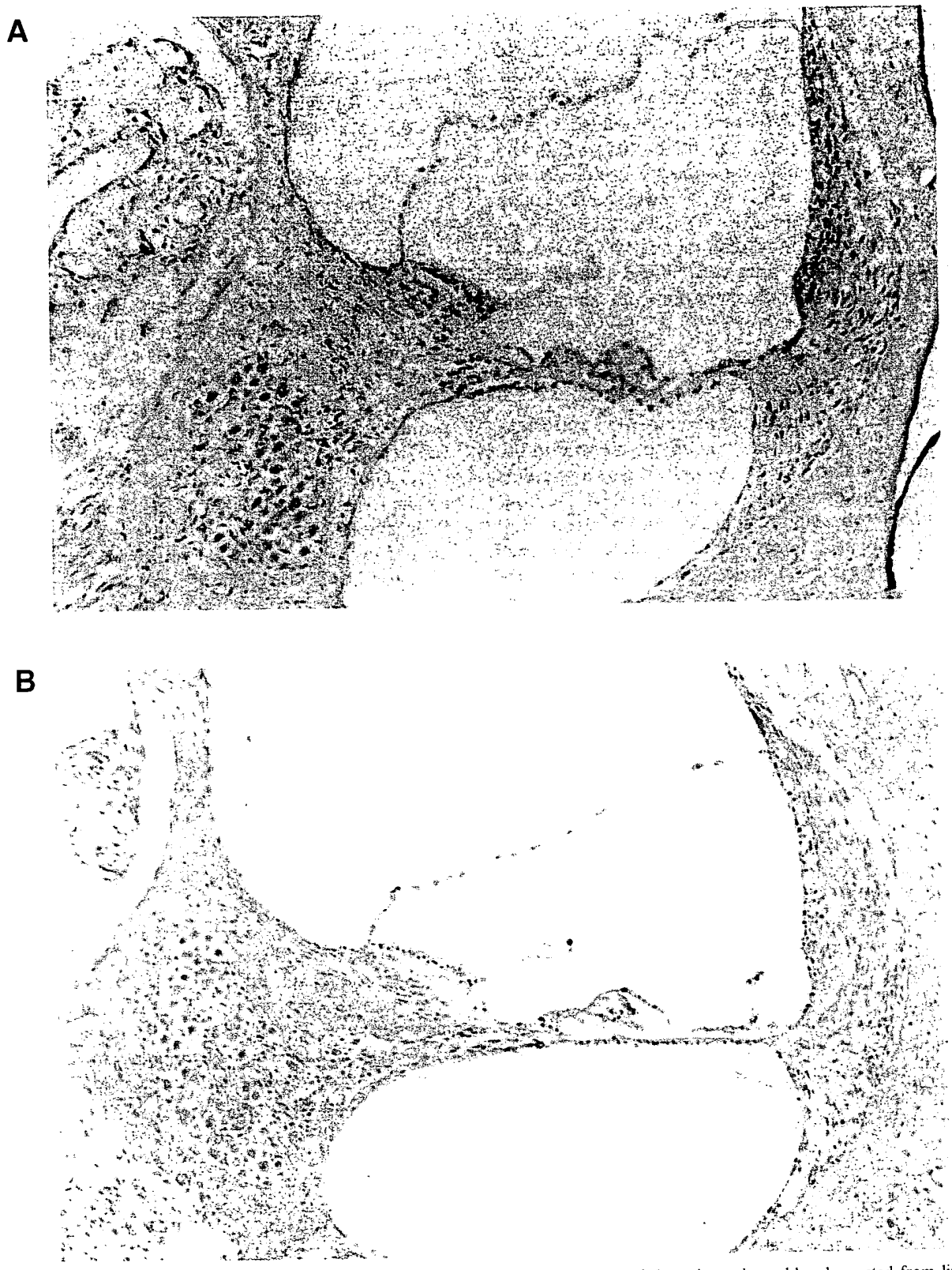

Fig. 1. Liposome mediated β-gal expression within the guinea pig cochlea. Radial sections of the guinea pig cochlea, harvested from liposome-β-gal or liposome injected animals were hybridized with β-gal antibody and then developed using a biotin labeled 2^0 antibody, Vector ABC amplification system and then stained with DAB. The cochleae were harvested from liposome-β-gal injected animals sacrificed at seven (A), and 14 (B and C) days post-injection or from liposome injected animals (D). While the liposome-injected cochlea is free of β-gal immunoreactivity, liposome-β-gal injected cochleae reveal β-gal immunoreactivity in several different tissue types that surround the cochlear duct. The transfected tissues include the spiral ligament, spiral limbus, the organ of Corti, Reissner's membrane and the spiral ganglia. The organ of Corti seen in B is illustrated at a higher magnification in C.

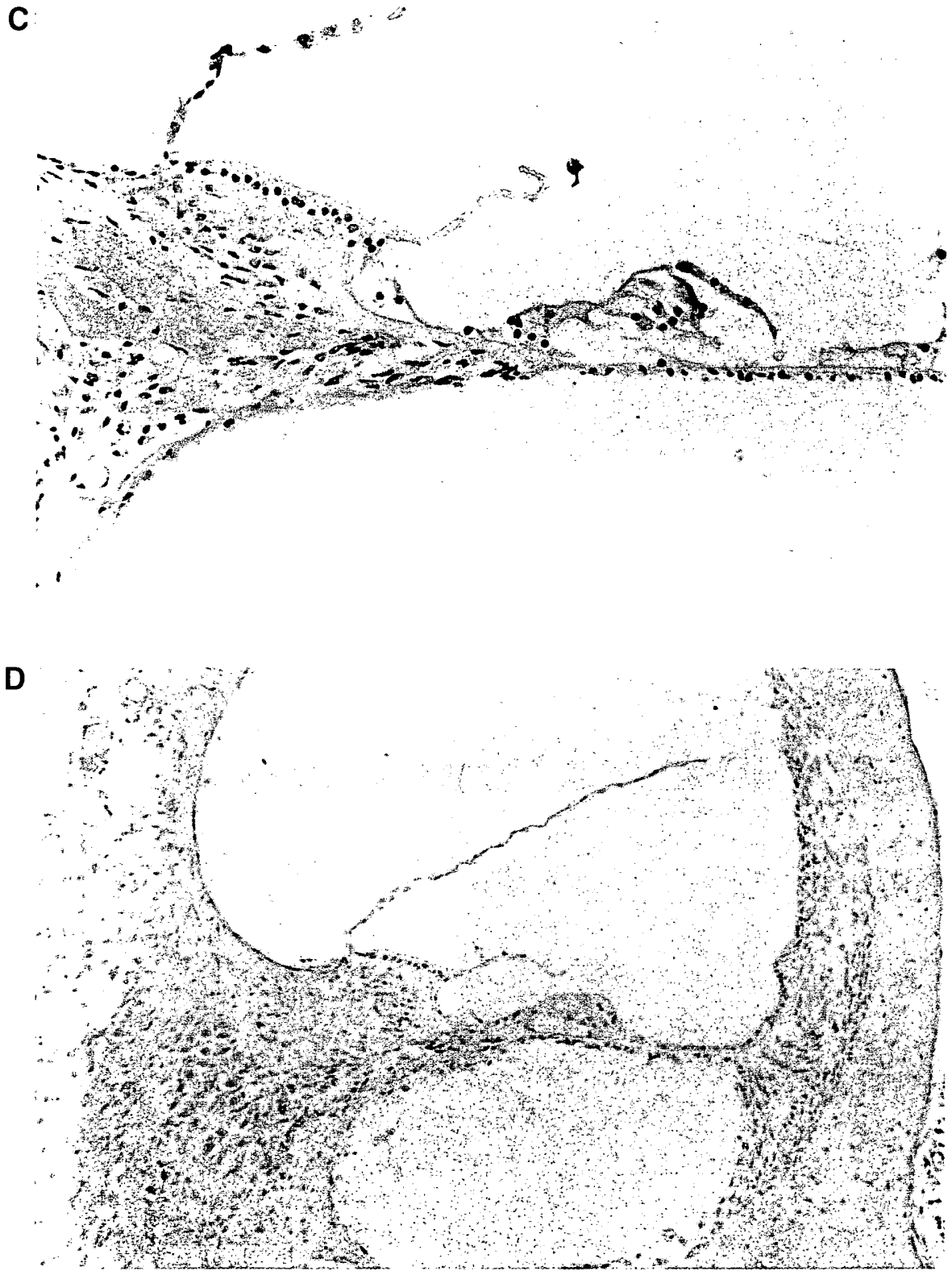

Fig. 1 (*continued*).

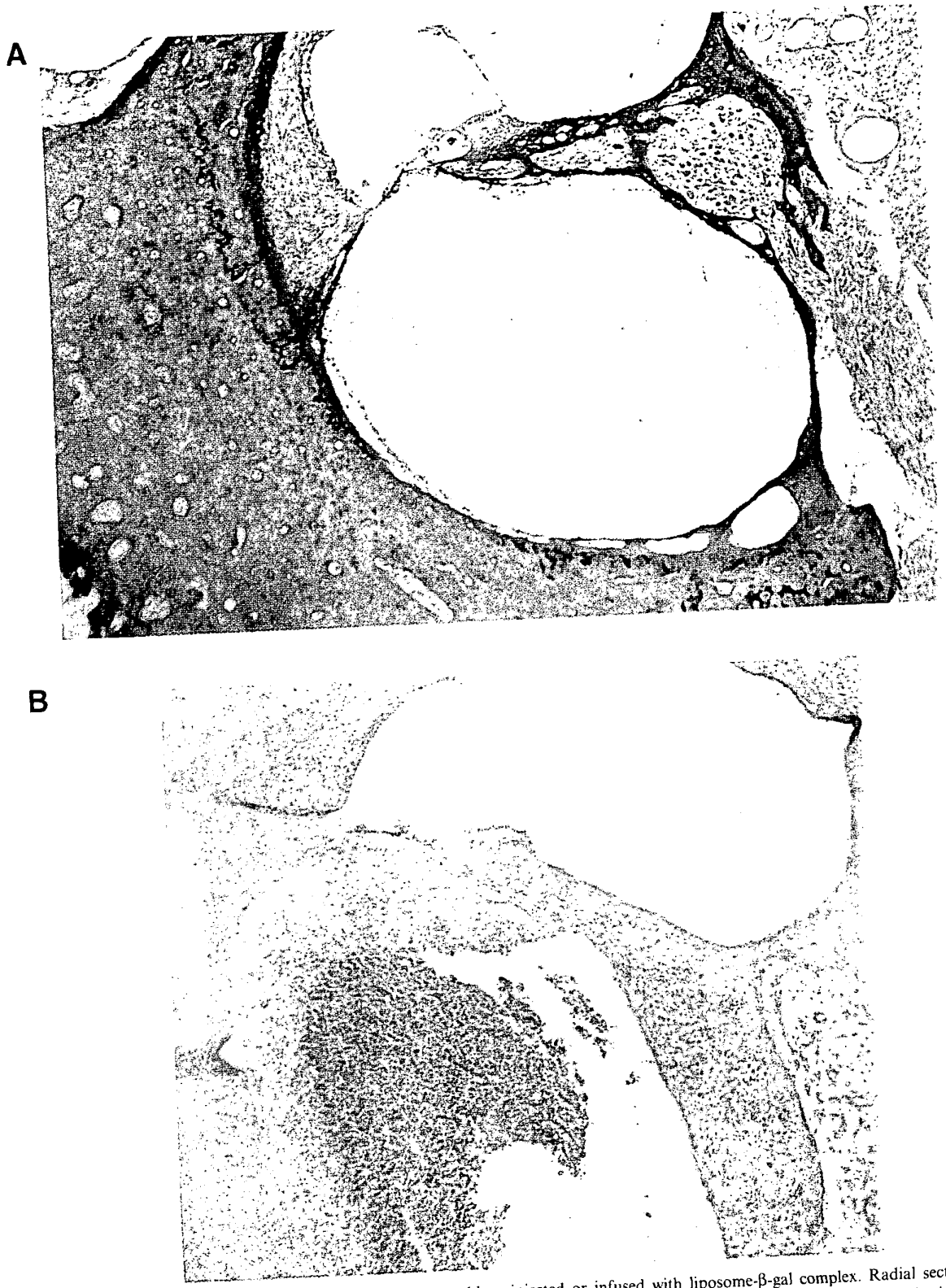

Fig. 2. Immunohistochemical analysis of immune response in cochleae injected or infused with liposome-β-gal complex. Radial sections of the guinea pig cochlea harvested at 6 days following intracochlear injection or infusion of liposome-β-gal complex were hybridized with anti-LA1 antibody. The bound antibody was detected using a biotin labeled 2^0 antibody, Vector ABC amplification system and then stained with DAB. Cochlear sections from injected animals show intact cytoarchitecture and were non-reactive with anti-LA1 antibody (A). Cochlear sections from infused animals show significant fibrosis at the site of catheterization and immunoreactivity towards the anti-LA1 antibody (B).

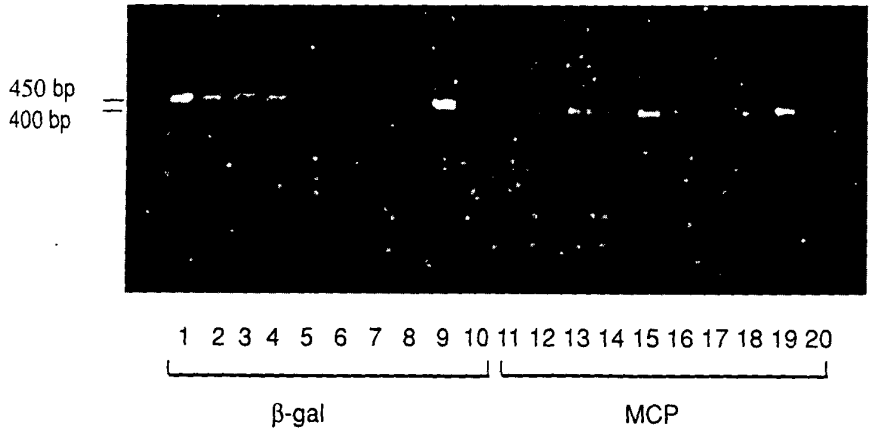

Fig. 3. PCR detection of the β-gal transgene. DNA extracted from paraffin embedded cochlear sections (used for immunohistochemical detection of β-gal) was amplified using either β-gal specific primers or for the gene encoding the membrane component protein (MCP) primers. The PCR product was resolved on a 1% agarose gel and detected using fluorescent ethidium bromide stain. DNA from the liposome-β-gal infused cochlear sections, harvested at 1, 3, 7 and 14 days post-injection (lanes 1, 2, 3 and 4), but not the corresponding contralateral sections (lanes 5, 6, 7 and 8) yield a β-gal-specific size fragment of 450 bp. The presence of genomic DNA in all samples assayed is reflected by the guinea pig genome-specific 400-bp fragments generated using guinea pig MCP primers (lanes 11–18).

photomicrograph. The brown DAB stain is present within the intact OHC as well as the supporting cells indicating marker gene expression within these cell types and detected via anti-β-gal antibody. Although the transgene expression persists 14 days post-injection (Fig. 1B), the intensity of β-gal immunoreactivity is relatively greater in the tissue sections from cochleae harvested at 7 days than 14 days post-injection (Fig. 1A and B). The relative decrease in immunoreactivity with time is indicative of a corresponding decrease in expression level of the transgene product. Cochlear sections of animals infused with liposomes (devoid of the transgene, β-gal) as well as saline were free of β-gal immunoreactivity (Fig. 1C). The contralateral cochleae of liposome-β-gal infused animals were also free of β-gal immunoreactivity (data not shown).

3.2. *Effect of the mode of delivery of the liposome-transgene complex upon cochlear cytoarchitecture and host-mediated immune response*

In addition to illustrating β-gal immunoreactivity, the photomicrographs in Fig. 1 also demonstrate relatively intact cochlear cytoarchitecture. These representative cochlear sections show no apparent signs of cellular degeneration or toxicity due to either the mode of vector delivery to the cochlea or the liposome/gene transfer vector or the transgene/transgene product. The cochlear sections from micro-injected animals were also characterized for immediate/acute inflammatory response. Antibodies to the T cell antigen, CD 45, and to the monocyte/macrophage antigen, LA1, were non-reactive against tissue sections from cochleae injected with liposome-β-gal (Fig. 2A).

Unlike the cochleae from micro-injected animals, cochleae from animals infused with liposome-β-gal via an osmotic mini-pump revealed significant fibrosis and acute immune response localized at the site of cochleostomy. Although non-reactive with Abs to the T cell antigen, CD 45, immunoreactivity to the monocyte/macrophage antigen, LA1, was detected in the tissue sections from liposome-β-gal infused cochleae (Fig. 2B).

3.3. *PCR detection of the transgene (β-gal expression plasmid)*

The presence of the transgene within the ipsilateral and contralateral cochleae of β-gal infused animals was separately assessed via a PCR-based assay. DNA was extracted from paraffin embedded cochlear sections (used for immunohistochemical detection of β-gal) and then amplified using either β-gal specific primers or for the gene encoding the membrane component protein (MCP) primers. The PCR products were resolved on a 1% agarose gel and the results illustrated in Fig. 3. A β-gal-specific size fragment of 450 bp was observed from DNA extract of the ipsilateral (liposome-β-gal infused) cochlear sections, harvested at 1, 3, 7 and 14 days post-injection (lanes 1, 2, 3 and 4), but not the corresponding contra-lateral sections (lanes 5, 6, 7 and 8). The presence and relative equivalency of genomic DNA amongst the extracts is reflected by the presence of equally intense 400-bp fragments generated using primers for the gene encoding the guinea pig membrane component protein (MCP) (lanes 11–18).

4. Discussion

Cochlear gene therapy studies have exclusively uti-

lized viral vectors for gene delivery. Although these studies have demonstrated feasibility of expressing transgenes within cochlear tissues, they have also exposed several major limitations associated with the use of viral vectors. These limitations include substantial time and effort involved in the preparation of the recombinant viral vectors and the potential safety hazards associated with their use. This study demonstrates the feasibility of using cationic liposomes to introduce exogenous genes directly within a mammalian cochlea, in vivo. Intracochlear injection or infusion of liposome-pCMV-β-gal complex, through the perilymph of scalae tympani, was shown to result in transfection and expression of β-gal in a number of different tissue types within the cochlea. Expression of the transgene product, β-gal, was shown to persist for up to two weeks following intracochlear infusion of the transfection complex. In addition, the cochleae of animals microinjected with the liposome-pCMV-β-gal complex were free of inflammatory response and cytotoxicity. The results of this study represent the first documented use of the cationic liposomes to transfect and express a transgene within cochlear tissues.

The cochlear tissue types that expressed the transgene include the spiral ligament, spiral limbus, spiral ganglia as well as the organ of Corti. Although the cationic liposomes are not known to differentiate between cell types, particular cell types do show relatively higher expression of the transgene while other cell types appear to be refractory to transfection. Type I and type II fibrocytes of the spiral ligament revealed the highest level of the transgene expression. Also of importance is the liposome mediated transgene expression within the cells of the organ of Corti, including the OHC. On the other hand, stria vascularis, an extensively vascular tissue type that forms the lateral boundary for the endolymph and is considered to be critical in the maintenance of its ionic balance, was devoid of transgene expression. The apparent absence of transfection in stria vascularis may result from exclusion of the transfection complex from the endolymph. However, not all tissue types that are transfected, e.g., the spiral ganglia, are directly accessible from the perilymphatic circulation into which the transfection complex is introduced. Cochlear microcirculation represents a potential migratory route for the dissemination of the transfection complex to the tissues distant from the perilymph. It is also possible that the transfection complex reached its target sites via simple diffusion from the site of its introduction. Consistent with this possibility is the observation of the transgene expression gradient that is highest at the base of the cochlea and diminishes towards the apex.

The cochlear tissue types transfected by the liposome-β-gal complex as well as the transgene expression gradient are similar to those observed using the AAV as the gene transfer vector (Lalwani et al., 1996). Unlike the AAV mediated transgene expression in the infused (ipsilateral) and the contralateral cochleae, liposome mediated β-gal expression was detected in the infused (ipsilateral) and not the contralateral cochlea. Absence of the reporter gene within the contralateral ear was confirmed independently via PCR analysis of extracts from the contralateral cochlear sections. The route of intercochlear migration by the AAV is unknown, although major pathways include cerebrospinal fluid space, connected to the perilymphatic fluid via the cochlear aqueduct, hematogenous circulation, and the bone marrow space of the temporal bone. The absence of liposome-β-gal complex within the contralateral cochlea may be a consequence of its larger size (260–560 nm in diameter) that may preclude its intercochlear dissemination as observed with AAV (15–20 nm in diameter). The restriction of its passage to distant tissue sites such as the contralateral cochlea may represent a potentially beneficial safety attribute of the cationic liposomes as gene therapy vectors.

Although the cochlear tissue or cell types transduced by AAV or transfected by cationic liposomes are similar, the level of transgene expression attained by these vectors remains to be determined. The similar transgene expression pattern of the cochlear tissues with cationic liposomes and AAV despite the relatively greater transduction efficiency of the former may be largely due to dosage effect. Approximately 10^{10} CMV-β-gal plasmid copies complexed with the cationic liposomes were delivered to the cochlea in the current study relative to 10^5 units of infectious AAV particles delivered to the cochlea (Lalwani et al., 1996).

Expression of the liposome-introduced transgene product was observed up to two weeks post-injection. Demonstration of persistence of the plasmid template within the tissue sections from cochleae harvested two weeks post-liposome-β-gal injection strongly refutes the possibility of the translated product persisting longer than its coding template. Persistence of the β-gal plasmid in tissue sections from cochleae harvested two weeks post-liposome-β-gal injection also suggests potentially longer duration of in vivo transgene expression. Presence of an eukaryotic origin of replication site within the pCMV-β vector enables its replication within eukaryotic cells and may contribute to its persistence in the transfected cells.

Mode of delivery to the target site as well as gene transfer vector or the transgene product may individually or collectively contribute to host-mediated local cytotoxicity and/or inflammatory response. Histopathological and immunohistochemical analysis of cochlea microinjected with the gene transfer vector revealed a relatively intact cytoarchitecture and absence of inflammatory response. Absence of cytotoxicity or inflammatory response in cochlea microinjected with the lipo-

some-β-gal complex suggests that the host may be able to sustain an increased or a multiple dose of the transfection complex, although physiologic effects of repeated administration of the liposomes are not known. Pump-mediated infusion of the gene transfer vector was shown to cause localized trauma and acute inflammatory response at the site of catheterization. The 10-fold greater volume of the transfection complex that is infused by the pump relative to micro-injection into the cochlea does not appear to yield greater expression of the transgene. The results of this study support the use of micro-injection over pump-mediated infusion as the efficacious mode of delivery of the gene transfer vector to the cochlea.

The successful use of the cationic liposomes as gene transfer vectors for the cochlea provides a safe and rapid alternative to the use of recombinant viral vectors for cochlear gene transfer studies. Liposome mediated gene transfer within the cochlea provides a rapid and useful strategy for functional analysis of the number of genes within the inner ear.

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After 24 hours, the cells were washed and cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum plus antibiotics (during the first 2 days with 1 mg/ml of G418 alone; subsequently, with G418 plus 1 mg/ml of zeocin). Colonies resistant to both antibiotics were picked, amplified, and screened for Env-dependent fusion permissiveness in the vaccinia assay system (with the use of luciferase as the reporter).
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52. Y.F. is a recipient of a National Research Council Research Associate fellowship, and C.C.B. is a recipient of an NIH Intramural Research Training Award. Support was provided by the NIH Intramural AIDS Targeted Antiviral Program. We are grateful to P. Clapham and R. Weiss, Chester Beatty Laboratories, Institute for Cancer Research, London, for donation of the CD4+ transformant of Mv 1 Lu; to M. Garfield, J. Lukszo, and J. Coligan, Laboratory of Molecular Structure, NIAID, for synthetic peptide synthesis, purification, and coupling; and to J. Sisler, Laboratory of Viral Diseases, NIAID, for oligonucleotide synthesis and assistance with DNA sequencing. We thank P. Murphy, NIAID, and B. Moss, NIAID, for helpful discussions and comments on the manuscript.

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Requirement of $p27^{Kip1}$ for Restriction Point Control of the Fibroblast Cell Cycle

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Cells deprived of serum mitogens will either undergo immediate cell cycle arrest or complete mitosis and arrest in the next cell cycle. The transition from mitogen dependence to mitogen independence occurs in the mid- to late G_1 phase of the cell cycle and is called the restriction point. Murine Balb/c-3T3 fibroblasts deprived of serum mitogens accumulated the cyclin-dependent kinase (CDK) inhibitor $p27^{Kip1}$. This was correlated with inactivation of essential G_1 cyclin-CDK complexes and with cell cycle arrest in G_1. The ability of specific mitogens to allow transit through the restriction point paralleled their ability to down-regulate p27, and antisense inhibition of p27 expression prevented cell cycle arrest in response to mitogen depletion. Therefore, p27 is an essential component of the pathway that connects mitogenic signals to the cell cycle at the restriction point.

Cells can respond to the absence of mitogenic signals and shift from a proliferating state to a quiescent state only during a brief window of time in the cell cycle. The transition from mitogen dependence to mitogen independence occurs during G_1 and has been called commitment (*1*). Many different antimitogenic signals cause the cell cycle to arrest at the same relative position in G_1, and cells become refractory to all of these signals at approximately the same time in mid- to late G_1 (*2*). This was named the restriction point, extending the original notion of commitment to include cellular responses to a variety of mitogenic cues. Time-lapse cinematography of mitotically proliferating single cells has confirmed that mitogen depletion causes cells in early G_1 to immediately exit the cell cycle, and that cell cycle commitment (autonomy from mitogenic signals) occurs in mid-G_1 (*3*). Thus, the proliferative effects of mitogenic signals are dependent on cell cycle position and are likely to be mediated by their action on proteins that control movement through the cell cycle.

Transit through G_1 and entry into the S phase requires the action of cyclin-dependent kinases (CDKs) (*4*), and CDKs are inactivated by growth inhibitory signals (*5*). The catalytic activity of CDKs is regulated by two general mechanisms, protein phosphorylation and association with regulatory subunits, including the cyclins and the CDK inhibitors (CKIs) (*6*). The CKI directly implicated in mitogen-dependent CDK regulation is $p27^{Kip1}$ (*7*). Amounts of p27 increase in quiescent cells and rapidly decrease after stimulation with specific mitogens (*8*). Moreover, constitutive expression of p27 in cultured cells causes cell cycle arrest in G_1 (*7*). Thus, p27 regulation may be an essential step in the pathway that links mitogenic signals to cell cycle progression and may be a key molecular event in the physiological process of cell cycle commitment or passage through the restriction point.

Subconfluent, exponentially proliferating Balb/c-3T3 fibroblasts [retinoblastoma (Rb) wild type; p53 status unknown] transferred to medium containing low concentrations of serum mitogens arrest in G_1 within 24 hours, which is approximately the length of one cell cycle (Fig. 1A). This demonstrates that Balb/c-3T3 cells require a mitogenic signal to proceed through each division cycle. G_1 arrest correlated with a six- to eightfold increase in the amount of the $p27^{Kip1}$ protein (Fig. 1B). Similar increases in p27 expression occur in primary human diploid fibroblasts deprived of serum mitogens and in primary human T lymphocytes after withdrawal of interleukin-2, indicating that this is a common pattern of p27 expression in nontransformed cells (*8*). In Balb/c-3T3 cells, the amount of p27 started to increase within 4 hours of serum withdrawal, reached 60% of maximal amounts within 12 hours, and peaked within 24 hours (*9*). The induction of p27 protein paralleled the accumulation of the initially asynchronous cell population in G_1 and was consistent with the hypothesis that it could play a critical role in the early events associated with exit from the cell cycle.

In accord with this conclusion, cell cycle arrest of Balb/c-3T3 cells correlated with down-regulation of the cyclin E-Cdk2 and cyclin A-Cdk2 protein kinases (*9*), and this appeared to be related to induction of p27. First, both cyclin E-Cdk2 and cyclin A-Cdk2 were associated with increased amounts of p27 after mitogen withdrawal (Fig. 2C). Second, immune-depletion experiments showed that only a small portion of cyclin E in proliferating cells was bound to p27,

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whereas all of the cyclin E in arrested cells was bound to p27 (Fig. 1C). Similar results were obtained for cyclin A (*10*).

We examined the abilities of specific serum mitogens to both down-regulate p27 and induce cell proliferation. Subconfluent proliferating Balb/c-3T3 cells were transferred to medium containing low concentrations of serum supplemented with single mitogens: platelet-derived growth factor BB (PDGF-BB), epidermal growth factor (EGF), or insulin-like growth factor 1 (IGF-1). Only PDGF-BB was sufficient to prevent G_1 arrest and induction of p27 (Fig. 1, A and B; Table 1). When grown at high cell density, no single mitogen is sufficient to cause proliferation. Instead, PDGF initially stimulates the density-arrested quiescent cells to become competent to respond to progression factors IGF-1 and EGF (*11*). Thus, passage through the restriction point does not occur until cells have been exposed to all three mitogens. In density-arrested cells, PDGF-BB alone was insufficient to alter the abundance of p27; rather, p27 amounts declined once cells became committed to proliferate in response to the complete mitogenic signal provided by the combined action of PDGF-BB, EGF, and IGF-1 (*12*). Thus, under two different growth arrest conditions, the ability of specific mitogens to stimulate passage through the restriction point correlated with their ability to regulate p27. Decreased amounts of p27 reflected the integrated action of the collection of mitogens required for cell proliferation.

We used antisense oligonucleotides to block expression of the p27 protein and thereby determine whether regulation of

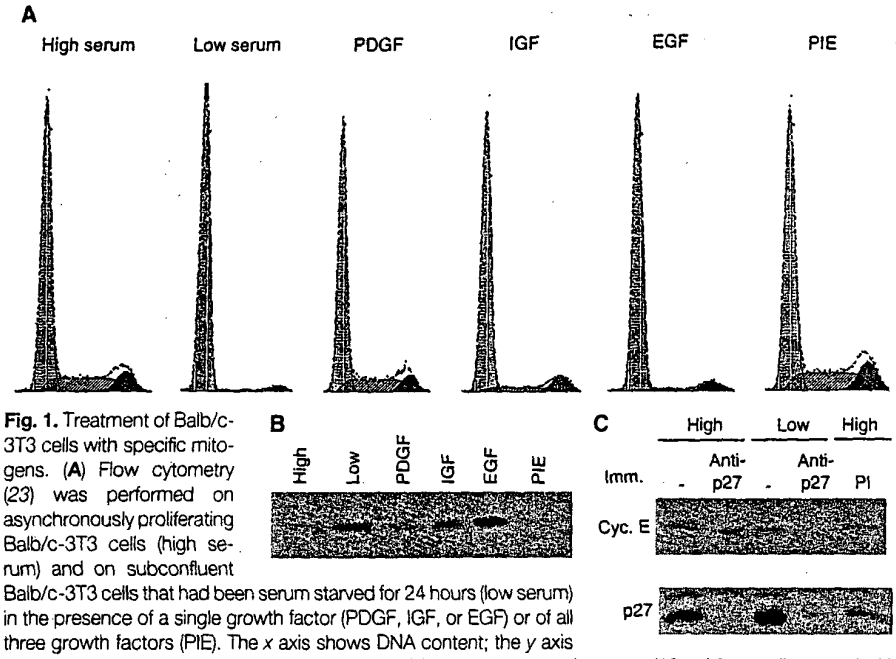

Fig. 1. Treatment of Balb/c-3T3 cells with specific mitogens. (**A**) Flow cytometry (*23*) was performed on asynchronously proliferating Balb/c-3T3 cells (high serum) and on subconfluent Balb/c-3T3 cells that had been serum starved for 24 hours (low serum) in the presence of a single growth factor (PDGF, IGF, or EGF) or of all three growth factors (PIE). The *x* axis shows DNA content; the *y* axis shows the number of cells. (**B**) p27 immunoblots (*8*) were done on cell extracts (10 μg) from cells treated with growth factors as indicated. (**C**) Cell extracts from asynchronously proliferating Balb/c-3T3 cells (high) and from Balb/c-3T3 cells that had been serum starved (low) for 24 hours were depleted of p27. Extracts (100 μg) were incubated with antiserum to p27 (anti-p27) and protein A agarose for 1 hour at 4°C and collected by centrifugation. The supernatant was incubated twice more with p27 antiserum and protein A agarose. The immune-depleted (imm.) extracts (anti-p27) were analyzed by immunoblotting with antibodies to cyclin E (cyc. E) (*22*) or p27 and were compared with undepleted extracts (−) and with extracts depleted with p27 preimmune sera (PI).

Table 1. Cell cycle analysis. Balb/c-3T3 (*14, 16*) and SAOS-2 (*14*) cells were treated with antisense (AS) or mismatch (MSM) oligonucleotides as described. High and low designate cells grown in 10% or 0.1% serum, respectively. The cell cycle distribution of cells treated with single growth factors (PDGF-BB, IGF, or EGF) is also shown. The data are presented as the percentage of cells in each phase of the cell cycle as determined by flow cytometry (*23*).

Cell type and condition	G_1	S	G_2/M
	Balb/c-3T3		
High serum	63.7	27.4	8.9
Low serum	86.9	9.3*	3.9
MSM-low	81.7	11.6	6.7
AS-low	62.2	23.4	14.4
MSM-high	59.2	26.8	14.1
AS-high	42.3	35.1	22.6
PDGF-BB	69.4	21.4	9.2
IGF-1	83.2	7.7	9.1
EGF	90.5	3.4	6.1
PDGF–IGF-1–EGF	64.2	23.8	11.9
	SAOS-2		
High serum	54.3	25.8	19.9
Low serum	70.6	13.6	15.8
MSM-low	60.5	16.8	22.7
AS-low	44.2	27.9	27.9

*Flow cytometry analysis overestimated the percentage of cells in the S phase. BrdU staining demonstrated that under low serum conditions, 2 to 5% of the cells were in the S phase.

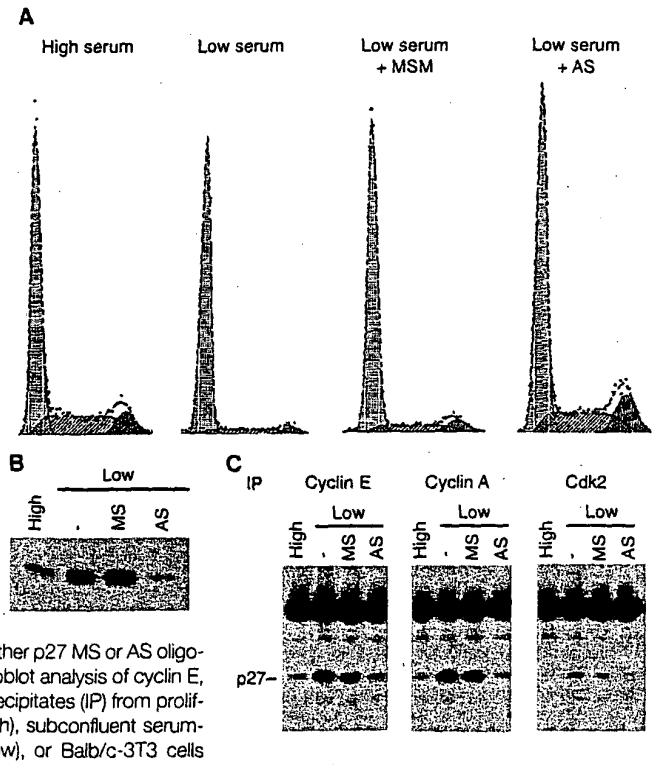

Fig. 2. Requirement of p27 for cell cycle withdrawal. (**A**) Flow cytometry analysis of proliferating Balb/c-3T3 cells (high serum), subconfluent Balb/c-3T3 cells that had been serum starved for 24 hours (low serum), or subconfluent Balb/c-3T3 cells that had been serum starved for 24 hours after lipofection with either p27 MSM or p27 AS oligonucleotides (*14*). *x* axis, DNA content; *y* axis, number of cells. (**B**) p27 immunoblot analysis of extracts (10 μg) from control proliferating Balb/c-3T3 cells (high), subconfluent serum-starved Balb/c-3T3 cells (low), and subconfluent Balb/c-3T3 cells that had been serum starved for 24 hours after lipofection with either p27 MS or AS oligonucleotides. (**C**) p27 immunoblot analysis of cyclin E, cyclin A, or Cdk2 immunoprecipitates (IP) from proliferating Balb/c-3T3 cells (high), subconfluent serum-starved Balb/c-3T3 cells (low), or Balb/c-3T3 cells that had been serum starved for 24 hours after lipofection with either MS or p27 AS oligonucleotides.

p27 was necessary for cell cycle control by serum mitogens. Phosphorothioate oligonucleotides were modified by the addition of a propynl group to the pyrimidine bases, which is thought to enhance base stacking and facilitate the sense-antisense interaction (*13*). Oligonucleotides were efficiently delivered to cells by association with a cationic lipid, GS2888 cytofectin. We used fluorescein isothiocyanate–labeled oligonucleotides to show that 90 to 95% of the cells took up and concentrated the oligonucleotides in the cell nucleus (*14*). We used two different 15-base antisense oligonucleotides directed against murine p27, and two different mismatch control oligonucleotides, which had the same base composition as the antisense oligonucleotides but a scrambled nucleotide sequence (*14*).

Balb/c-3T3 fibroblasts were exposed to p27 antisense and mismatch control oligonucleotides and then transferred for 24 hours to medium lacking serum mitogens. Identical results were obtained with both antisense or with both control oligonucleotides. The expression of p27 protein was substantially decreased in the antisense-treated cells (Fig. 2B), whereas the mismatch control had no effect on accumulation of p27 after serum withdrawal. Treatment of cells with antisense oligonucleotides to p27 did not decrease expression of the related CKI, $p21^{CIP1}$ (*15*). A decrease in the association of p27 with cyclin A and cyclin E corresponded to the decrease in overall amount of p27 in the antisense-treated cells (Fig. 2C). This was associated with restoration of cyclin E– and cyclin A–associated kinase activities in serum-starved cells (9).

In a proliferating population of Balb/c-3T3 fibroblasts, 27% of the cells were in S phase, and this fell to about 9% of cells within 24 hours after serum withdrawal (Fig. 2A, Table 1). Cells exposed to the mismatch oligonucleotide behaved identically to control cells. However, cells exposed to p27 antisense oligonucleotides did not undergo G_1 arrest after serum withdrawal; 23% of the cells remained in S phase (Fig. 2A, Table 1). The p27 antisense oligonucleotides also prevented the osteosarcoma cell line SAOS-2 (Rb mutated; p53 mutated) from exiting the cell cycle in response to serum withdrawal (Table 1). Thus, p27 is required for mitogen responsiveness in more than one cell type, and the requirement is independent of the Rb status of the cell.

Incorporation of bromodeoxyuridine (BrdU) or tritiated thymidine into nuclear DNA was used as an independent measure of the effect of p27 antisense oligonucleotides on cell cycle progression (*16*). Both techniques showed that cells exposed to p27 antisense oligonucleotides continued to synthesize DNA for at least 24 hours after serum withdrawal, whereas cells treated with mismatch control oligonucleotides did not (Fig. 3C) (*16*). Although the duration of antisense p27 inhibition is limited, cells treated with p27 antisense oligonucleotides express low amounts of p27 protein and continue to proliferate for at least 48 hours without serum mitogens.

To demonstrate the specificity of the antisense oligonucleotides (*17*), we showed that enforced expression of p27 in antisense-treated cells restored serum responsiveness. The degeneracy of the genetic code was exploited to construct a p27 expression plasmid that could not be inhibited by the antisense oligonucleotides but nevertheless encoded wild-type p27 protein (the p27 "wobble" plasmid) (Fig. 3A). A "tagged" version of the p27 wobble plasmid also was constructed, which encoded an electrophoretic variant of p27 resulting from a single amino acid change outside of the domain targeted by the antisense oligonucleotide. The tagged p27 could be separated and thereby distinguished from endogenous p27, enabling us to simultaneously test the effects of p27 antisense oligonucleotides on expression from the genes encoding the wild-type and wobble p27 in the same cell. The p27 antisense oligonucleotides effectively inhibited expression from both an exogenous wild-type gene for p27, and from the endogenous gene for p27, but did not inhibit p27 protein expression from the p27 wobble plasmid (Fig. 3B). Balb/c-3T3 cells were lipofected with mismatch or p27 antisense oligonucleotides and then microinjected with a plasmid encoding β-galactosidase (β-Gal) (to mark the injected cells) and the p27 wobble plasmid. Cells were then serum-starved for 24 hours, and the percentage of cells in S phase was measured by exposure to BrdU. Lipofection of cells with p27 antisense oligonucleotides decreased the percentage of cells that withdrew from the cell cycle after mitogen depletion, and this was reversed by microinjection with the p27 wobble plasmid (Fig. 3C). These results showed that the inability of p27 antisense-treated cells to exit the cell cycle after mitogen depletion is specifically caused by the loss of p27 expression.

It has been suggested that the basal amount of p27 expressed in proliferating cells may contribute to an inhibitory threshold imposed on CDK activation during G_1 (*18*). At one extreme, large amounts of p27 prevent CDK activation and arrest the cell cycle in G_1 (*7*), whereas decreased p27 expression might allow premature CDK activation and a shortened G_1 (*19*). To test this idea, exponentially proliferating Balb/c-3T3 cells were lipofected with p27 antisense or mismatch control oligonucleotides and allowed to proliferate in medium containing a high concentration of serum for an additional 24 hours. The p27 antisense treatment decreased p27 protein expression in proliferating cells to well below the normal basal amount. This markedly decreased the percentage of cells in G_1 (Table 1), showing that the length of G_1 was shortened relative to other phases of the cell cycle. No effect on p27 expression or cell cycle distribution was seen in the mismatch control (9). This is consistent with the hypothesis that the amount of p27 expressed in proliferating cells contributes to the length of G_1.

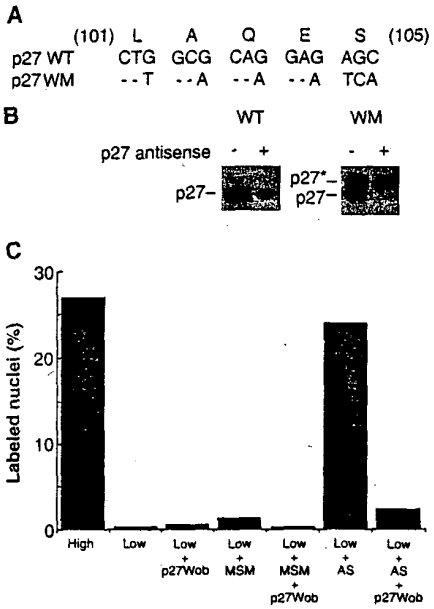

Fig. 3. Restoration of serum responsiveness by enforced expression of p27. (**A**) Depiction of bases that were changed in the p27 wild-type (WT) sequence to create the p27 wobble mutant (WM) plasmid (*28, 29*). These are the bases that hybridize to p27 antisense oligonucleotides. (**B**) p27 immunoblot analysis of proliferating Balb/c-3T3 cells 24 hours after lipofection in the presence (+) or absence (–) of p27 AS oligonucleotides with plasmid encoding either WT or the tagged (p27*) p27 wobble mutant (WM). (**C**) Proliferating Balb/c-3T3 fibroblasts (high) were lipofected with p27 MSM or AS oligonucleotides for 6 hours in a high concentration of serum. Cells were then microinjected with plasmids encoding β-Gal (*14*) and untagged p27 wobble mutant plasmid (p27Wob) as indicated. Cells were rinsed once with serum-free medium and incubated for 24 hours in medium containing 0.1% serum (low). Cells were labeled for the last 3 hours of the experiment with BrdU. Cells were stained for β-Gal expression (*14*) and immunostained for BrdU incorporation. The percentage of β-Gal–positive cells that incorporated BrdU was determined. Data represent the average of three independent experiments.

Our results show that restriction point control of nontransformed, immortalized Balb/c-3T3 cells requires the CDK inhibitor $p27^{Kip1}$. In cells deprived of mitogens, p27 amounts are elevated, essential cyclin-CDK complexes are inactivated, and the cell cycle stops in G_1. If p27 amounts do not increase, then the cell remains committed to the cell cycle independently of serum mitogens. As in the cells studied here, p27 expression is also mitogen dependent in primary cells and in other established cell lines. However, it remains possible that some regulatory pathways that contribute to restriction point control in vivo are no longer evident in some established cell lines.

Mitogenic signals have other effects on cell cycle regulators such as the Rb protein, whose phosphorylation by CDKs temporally coincides with the restriction point in G_1 (*20*). p27 is a global regulator of CDKs in G_1, so the control of Rb phosphorylation by mitogens could reflect the intermediary action of p27. Some aspects of the restriction point are altered in cells lacking Rb, such as the ability of the cell cycle to arrest after partial inhibition of protein synthesis (*21*), but $Rb^{-/-}$ mouse embryo fibroblasts remain responsive to mitogens. Rb phosphorylation may represent just one of a set of CDK-dependent events that occur at the restriction point. G_1 cyclin expression is also mitogen-dependent (*22*), and constitutive cyclin overexpression in cultured fibroblasts partially overcomes the antiproliferative effect of mitogen depletion (*19*). Thus, mitogens have complementary effects on CDK activation—they both increase expression of cyclins, the CDK-activating subunits, and decrease expression of p27, a CDK-inhibitory subunit. The concerted regulation of cyclins and CDK inhibitors allows transit through G_1 and is required to link mitogenic signals with cell cycle progression at the restriction point. How these pathways become independent of mitogens in post–restriction point cells remains to be determined.

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9. Proliferating Balb/c-3T3 fibroblasts were rinsed in serum-free medium and refed with medium containing 0.1% serum. p27 protein immunoblots (ECL, Amersham) were performed on cells harvested at 4, 8, 12, 16, and 24 hours after refeeding. Histone H1 kinase assays (*8*) were done on cyclin A, cyclin E, and Cdk2 (*23*) immunoprecipitated from Balb/c-3T3 extracts made from proliferating and serum-starved cells. Serum-starved cells lipofected with p27 antisense oligonucleotides contained increased amounts of cyclin E– and cyclin A–associated histone H1 kinase activity as compared with serum-starved cells. For experiments done with proliferating cells, proliferating cells were lipofected with either p27 mismatch or p27 antisense oligonucleotides and analyzed 24 hours later by flow cytometry and p27 immunoblots. The amount of p27 was three to five times less in cells treated with p27 antisense oligonucleotides than in proliferating cells and proliferating cells lipofected with p27 mismatch oligonucleotides.
10. Experiments were done as described (Fig. 1C), with the exception that cyclin A and p27 immunoblots were done on extracts depleted in p27. All of the cyclin A was bound to p27 in extracts from serum-starved cells, whereas only a small fraction (5%) of cyclin A was associated with p27 in proliferating cells.
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14. The oligonucleotides were synthesized on an automated synthesizer (model 8750, Milligen Bioresearch, Bedford, MA) as described (*24*). The antisense oligonucleotide sequence used in these experiments was 5′-UGG CUC UCC UGC GCC-3′ (targets base pairs 306 to 320 of murine Kip1) and the mismatch sequence was 5′-UCC CUU UGG CGC GCC-3′. For the lipofection procedure, 30 nM oligonucleotides were mixed with GS2888 cytofectin (2.5 μg/ml) (*25*) (Gilead Scientific, Foster City, CA) in serum-free medium and incubated for 10 min at 37°C. Proliferating Balb/c-3T3 fibroblasts were rinsed once in serum-free medium and refed with oligonucleotide-cytofectin solution in medium containing 0.1% serum. Cells were then incubated for 24 hours in humidified incubators at 37°C with 5% CO_2. The percentage of cells that were positive for uptake of fluorescein isothiocyanate–labeled oligonucleotides was determined by ultraviolet fluorescence microscopy. Microinjection, immunofluorescence staining, and fluorescence microscopy were carried out as described (*26*). For costaining of β-Gal and BrdU, the cells were fixed and stained as previously described (*26, 27*).
15. Proliferating Balb/c-3T3 fibroblasts were lipofected with antisense and mismatch oligonucleotides (*14*). The amount of p21 was increased in proliferating cells as compared with that in serum-starved cells (*23*). Cells lipofected with either p27 mismatch or antisense oligonucleotides expressed slightly larger amounts of p21 as compared with amounts in serum-starved control cells.
16. Proliferating Balb/c-3T3 fibroblasts were lipofected with p27 mismatch or antisense oligonucleotides (*14*). For BrdU experiments, Balb/c-3T3 cells were labeled for the last 3 hours of the experiment with BrdU and immunostained with monoclonal antibodies to BrdU as described (*22*). The percentage of total cells (on a 1-mm cover slip) that stained positive for BrdU incorporation (percent of labeled nuclei) was determined. Thirty-five percent of serum-starved cells treated with p27 antisense oligonucleotides incorporated BrdU into nuclear DNA, whereas only 2 to 3% of the cells treated with mismatch control oligonucleotides did so. For tritiated thymidine experiments, cells were labeled for the last 3 hours of the experiment with [^{3}H]thymidine (1 μCi/ml). The amount of [^{3}H]thymidine incorporated into serum-deprived cells treated with antisense or mismatch oligonucleotides was compared with the amount of [^{3}H]thymidine incorporated into asynchronously proliferating cells.
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28. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; D, Asp; E, Glu; L, Leu; Q, Gln; and S, Ser.
29. To construct the p27 wobble expression plasmid used in Fig. 3C, a megaprimer was generated by polymerase chain reaction (PCR) amplification with the use of a primer to plasmid sequences (T7 primer) and a primer that contained mutations at the wobble positions for the amino acid sequence LAQESQ (*28*) (amino acids 102 to 108) of murine p27. (5′-TAA AGG CAC CGC CTG GCG ACT ACC GCT GAC GTC CTG TGA TTC TTG TGC AAG CAC CTT GCA GGC GCT C-3′). The megaprimer was subsequently used with a primer to plasmid sequences (T3 primer) at the 3′ end to PCR-amplify a full-length clone, which was subcloned into the expression vector pCS2+. These mutations created a p27 sequence with seven bases unmatched to the p27 antisense oligonucleotide and created a unique Aat II site. In addition to the base changes listed above for amino acids 102 to 108, the tagged p27 wobble mutant used in Fig. 3B also fortuitously contained mutations at Ser^{111} and Arg^{112}. These amino acids had been converted to Thr and Ser, respectively. Electrophoretically, the tagged p27 wobble mutant migrates slightly more slowly than do endogenous murine p27 and exogenous wild-type p27.
30. We thank members of the Roberts lab for suggestions and comments throughout the course of this work; R. Herrera, R. Weinberg, and T. Jacks for communicating results before publication; and D. Grant for technical assistance. Supported by a postdoctoral fellowship from the American Cancer Society (S.C.), an NIH Cancer Biology Training grant (J.N.), and a Howard Hughes Medical Institute (grant to G. Crabtree) and the NIH (J.R.).

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D E C L A R A T I O N

I, JOHN ALFRED RICHES, Fellow of the Institute of Linguists, of Oak Farm, Catfield, Great Yarmouth, Norfolk, England, do hereby declare that I am conversant with the English and German languages and am a competent translator thereof. I declare further that the following is a true and correct translation made by me of patent application PCT/EP99/01153 in the German language attached hereto.

Signed this 15th day of August, 2000.

DECLARATION

I, JOHN ALFRED RICHES, Fellow of the Institute of Linguists, of Oak Farm, Catfield, Great Yarmouth, Norfolk, England, do hereby declare that I am conversant with the English and German languages and am a competent translator thereof. I declare further that to the best of my knowledge and belief the following is a true and correct translation made by me of internation patent application PCT/EP99/01153.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Signed this fifteenth day of August, 2000.

PROCESS FOR THE TREATMENT OF DISEASES OR DISORDERS OF THE INNER EAR

DESCRIPTION

The invention firstly relates to a process for the treatment of diseases or disorders of the inner ear, which are linked with damage or destruction of the sensory cells of the inner ear.

The inner ear of humans and other mammals can either be irreversibly damaged from the outset by a genetic defect or subsequently by external influences. These external influences can e.g. be acoustic trauma or toxic or hypoxic influences. Such damage can lead to functional disturbances or losses of the senses located in the inner ear, particularly hearing. In the case of these functional disturbances particular reference must be made to a reduction or disappearance of the power of hearing. It is estimated that in Germany approximately 12 million people suffer from a so-called perceptive deafness, which can be attributed to the aforementioned pathogenetic mechanisms. Apart from the degeneration of sensory neurons and damage to the so-called stria vascularis of the inner ear, a cause of partial or complete loss of the power of hearing can in particular be damage or destruction of the sensory cells of the inner ear and consequently the hearing organ.

In a process for the treatment of diseases or disorders of the inner ear linked with damage or destruction of the sensory cells, it must be borne in mind that it is no longer possible to regenerate irreversibly damaged and therefore lost cells in the highly differentiated sensory epithelia in the inner ear of humans and other mammals. Thus, a partial or complete hearing loss due to damage or destruction of the sensory cells of the inner ear is generally irreversible. In this respect the sensory epithelia of the inner ear fundamentally differ from other tissues, where necrotic cells can be rapidly replaced by the division of substitute cells and their subsequent maturation.

It is of interest that in other vertebrate classes, such as e.g. birds, necrotic sensory cells in the inner ear can be regenerated, unlike the situation with humans. In birds sensory cells which have died after damage are replaced by so-called supporting cells located in the epithelium below the sensory cells. This takes place by division of the supporting cells and subsequent maturation, a new supporting cell and a sensory cell resulting from a supporting cell.

The discovery of the regeneration of sensory cells in the cochlea of the bird has over the past few years led to an attempt being made to transfer research results made on the bird to mammals and therefore ultimately humans. This inter alia promised success, because the cochlea of the bird and the cochlea

of mammals have cell-biological points in common. Both the sensory epithelium of bird cochlea and the sensor epithelium of mammal cochlea are postmitotic, i.e. sensory cells present in the sensory epithelia are formed only during a specific time period of embryonic development, after which normally no further cell divisions occur. However, this fundamental point in common makes it difficult to understand the phenomenon that in the vestibular sensory epithelium of the bird cell divisions can be detected throughout its life, but not in humans.

As it was recognized in the bird that so-called growth factors can give rise to an increased proliferation rate in the bird cochlea, such growth factors were also used in the mammal cochlea. However, it was not possible to prove a reproducible action. This makes it obvious to draw the conclusion that despite fundamental cell-biological points in common, there must be other significant differences between bird and mammal cochlea. These could be that the supporting cells of the bird cochlea, as in the mammal, are postmitotic, but have only temporarily left the cell cycle. They can then reenter the cell cycle when a corresponding signal appears. Such supporting cells can be called quiescent, i.e. they are in the waiting state. As opposed to this the supporting cells of the mammal pass through a very high and specific differentiation and consequently irreversibly leave the cell cycle. They can consequently be called terminally differentiated and are e.g. comparable with neurons. This can apply in the case of the supporting cells of the mammal, which are referred to as so-called Pillar's or Deiter's cells. Such explanation models for cell-biological differences between bird and mammal cochlea have given rise to a more detailed investigation of the regeneration of the sensory cells in the bird in order to subsequently transfer the results obtained to mammals.

However, the problem of the present invention is to find a new starting point for the treatment of disorders or diseases of the inner ear, which are linked with damage or destruction of the sensory cells of the inner ear. The aim is less to transfer to mammals and in particular humans results obtained on vertebrates other than mammals and more to make available an action mechanism and corresponding active ingredients, which act directly in the cellular processes in the mammal and ultimately lead to a regeneration of the sensory cells of the inner ear.

This problem is solved by the process having the features of claim 1 and the process with the features of claims 2 and 3. Preferred developments are described in the dependent claims 4 to 21. Thus, by reference, the content of all the claims is made into part of the present description.

According to the invention, the process of the aforementioned type is

characterized in that at least one so-called cell cycle inhibitor present in the inner ear has its inhibiting action partly inhibited or eliminated by at least one active ingredient, which results in a regeneration of the sensory cells of the inner ear. From the patent law sense this process also incorporates the use of an active ingredient able to inhibit or eliminate the action of a cell cycle inhibitor present in the inner ear, either directly for the treatment of diseases or disorders of the inner ear or indirectly for preparing a pharmaceutical composition or a medicament for the treatment of diseases or disorders of the inner ear, said diseases/disorders being linked with damage or destruction of the sensory cells of the inner ear.

The regeneration of the sensory cells of the inner ear resulting from the process according to the invention preferably takes place through a stimulation of the proliferation of the supporting cells of the inner ear, i.e. the supporting cells also present in the sensory epithelium and usually located between and below the sensory cells. As there are one or more cell cycle inhibitors in the supporting cells of the inner ear, by inhibiting or eliminating their inhibiting action by a suitable active ingredient it is possible to initiate the cell division of the supporting cells, thereby creating a fundamental prerequisite for creating replacement or substitute cells for the necrotic or dead sensory cells. The cells resulting from the division of the supporting cells can then at least partly mature to functional sensory cells.

With regards to the sensory cells of the inner ear referred to up to now, these are preferably so-called hair sensory cells or short hair cells, which have at their upper end hair-like extensions, so-called stereocilia or small sensory hairs. These hair cells are located on the basilar membrane in the so-called corti-organ and form as so-called outer and inner hair cells the actual receptor cells for acoustic transduction in the inner ear. Both the inner and the outer hair cells are of interest for regeneration, regeneration of the outer hair cells representing a particular field of use of the invention as a result of their greater sensitivity. Those supporting cells which are anatomically particularly well associated with the inner or outer hair sensory cells can in particular be used for the active ingredient employed according to the invention. Thus, apart from outer hair sensory cells as supporting cells can be used the so-called Hensen's cells and, below the outer hair sensory cells, the so-called Deiter's cells and "outer" Pillar's cells. These Hensen's, Deiter's and outer Pillar's cells are consequently particularly suitable as replacement cells for the outer hair sensory cells. Correspondingly alongside and below the inner hair sensory cells are provided the so-called inner sulcus cells as supporting cells and within the inner hair sensory cells also the inner Pillar's cells, both being usable as replacement cells for the inner hair sensory cells. Thus, optionally a regeneration of inner or outer hair cells can be selectively initiated

and influenced. Reference can be made in this connection to the relevant textbooks and articles concerning the hearing process in mammals, particularly humans. The regeneration of the hair sensory cells participating in acoustic transduction in the inner ear for the treatment of perceptive deafness in the case of damage to said sensory cells represents the main field of use of the present invention.

The cell cycle inhibitors, whose inhibiting action is to be inhibited or eliminated according to the invention, can fundamentally be different physiological substances, particularly proteins, preventing the cell passing through the normal cell cycle, including cell division. They are preferably so-called cyclin-dependent kinase inhibitors (CDKIs). It is known that during the development of an organism they are expressed to a reinforced extent during the occurrence of terminally differentiated cells and in this way prevent the reentry of the cell into the cell cycle. This would also explain the loss of the dividability of such cells with reinforced expression of cyclin-dependent kinase inhibitors. Cell cycle inhibitors and in particular cyclin-dependent kinase inhibitors of the so-called CIP/KIP family can be selectively expressed in specific cell types. Preferred cyclin-dependent kinase inhibitors are in particular the proteins referred to as $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$. According to the invention preference is given to the cyclin-dependent kinase inhibitor $p27^{Kip1}$. As a result of the selective expression of such inhibitors and the different expression patterns resulting therefrom, the invention can be used for selectively influencing the cell cycle in a specific cell type. If e.g. in a specific cell type, such as e.g. the supporting cells in the sensory epithelium of the inner ear, $p27^{Kip1}$ is expressed selectively or at least with a significant proportion, by means of an active ingredient aimed specifically at this inhibitor, it is possible to eliminate its inhibiting action and consequently initiate or stimulate the proliferation of supporting cells. By means of a maturation of at least part of the cells resulting from the division of the supporting cells, a regeneration of the sensory cells is brought about.

As is apparent from the statements up to now, according to the invention the inner ear disease or disorder involved is in particular a so-called perceptive deafness. This is linked with the already described damage or destruction of the hair sensory cells in the inner ear.

In the case of the active ingredient usable according to the invention, which inhibits or eliminates the inhibiting action of the cell cycle inhibitor, is preferably a substance, which normally acts in intracellular manner either directly or indirectly on the inhibitor, i.e. normally a peptide or protein. The active ingredient is preferably present in the form of a peptide or protein, which effects a peptide-peptide or protein-protein interaction with

the inhibitor. This would then be the case of a "direct" influencing of the function of the inhibitor. If the active ingredient is constituted by a nucleic acid molecule, which codes one of the aforementioned peptides/proteins for the amino acid sequence, it is possible to refer to an "indirect" influencing, because initially the coding nucleic acid molecule is introduced into the corresponding cell and subsequently the peptide/protein molecule (serving directly as the active ingredient) is expressed. Said nucleic acid molecule can in particular be a recombined nucleic acid molecule. The nucleic acid molecule can fundamentally be a DNA molecule, a cDNA molecule or a RNA molecule.

Another active ingredient usable in preferred manner according to the invention is a nucleic acid molecule, where use is made of the so-called antisense method. In this method which is fundamentally known to the expert use is normally made of a RNA, which is complimentary to the RNA of the normal (physiological) gene. This complimentary RNA is called antisense-RNA. The antisense-RNA can prevent the synthesis of the protein product belonging to the gene. In the case of the invention this means that a nucleic acid molecule, e.g. the antisense-RNA itself or DNA, during whose transcription the antisense-RNA is formed, is introduced into the organism or cell for inhibiting or eliminating the inhibiting action of the cell cycle inhibitor. This introduction preferably takes place with the aid of lipid compounds, which also carry viral components for the better docking and penetration of the nucleic acid molecule into the cell.

As stated, the active ingredient in the case of the invention can effect a direct interaction, preferably a peptide-peptide or protein-protein interaction with the cell cycle inhibitor. However, the active ingredient can also indirectly inhibit or eliminate the inhibiting action of the cell cycle inhibitor, in that it interacts at least as well or preferably better with a physiological interaction partner of the cell cycle inhibitor than the cell cycle inhibitor itself. This prevents the cell cycle inhibitor from evolving its physiological (inhibiting) action.

Thus, e.g. in the case of the cyclin-dependent kinase inhibitor $p27^{Kip1}$, it is known that it forms a protein complex together with the cyclin-dependent kinase CDK2 and cyclin A. There are specific points at which peptide-peptide interactions occur between the $p27^{Kip1}$ and the CDK2 or cyclin A. Thus, e.g. identification has taken place of a bonding point of very high affinity between $p27^{Kip1}$ and cyclin A and several less strong bonding points between $p27^{Kip1}$ and cyclin A or $p27^{Kip1}$ and CDK2. On extracting one of the bonding points where there is no high or very high affinity bonding/interaction, an active ingredient, preferably in the form of a further peptide/protein can be selected or developed can effect a bonding/interaction of at least as high or

preferably higher affinity with one of the two interaction partners at the particular bonding point. This inhibits or prevents the standard physiological interaction, because the corresponding bonding point for the physiological interaction partner is blocked.

Thus, e.g. for a bonding point between $p27^{Kip1}$ and cyclin A, but also CDK2, an optimized peptide structure or optimized amino acid sequence can be developed for the amino acid sequence of $p27^{Kip1}$ at this point, which then bonds with a better, i.e. higher affinity with the corresponding sequence of cyclin A or CDK2 at this point. Such an optimized peptide structure e.g. and preferably comprises up to 15 amino acids and can then be directly introduced into the cell or preferably expressed in intracellular manner by an artificially introduced gene. Through the high affinity of such a peptide the interaction of the physiological peptide partner is then destroyed and the formation of the peptide complex, based on the inhibiting action of the cell cycle inhibitor is prevented. Thus, the active ingredient ensures an at least partial inhibition or a complete elimination of the inhibiting action of the cell cycle inhibitor. As a result of this process starting point of the invention the concentration of the active ingredient, particularly the peptide/protein with the corresponding amino acid sequence in the cell only has to be roughly of the same level as the corresponding concentration of the cell cycle inhibitor, whose action is to be inhibited or eliminated. As such concentrations, e.g. of $p27^{Kip1}$ are approximately 10 nM/l and roughly correspond to 1,000 to 10,000 molecules per cell, even very low concentrations can suffice for the performance of the invention. It is also important that for achieving such a concentration using gene-therapeutic methods it is sufficient to introduce only a single copy of a DNA, coding for the corresponding amino acid sequence, for each cell. Compared with other methods which have to use much higher concentrations or a larger number of DNA copies, this represents a decisive advantage.

According to a further development the process according to the invention can be performed in such a way that the active ingredient is in the form of a so-called vector or vehicle, said vector or vehicle carrying at least one of the above-described nucleic acid molecules. Preferably it is a nucleic acid molecule, which codes for the amino acid sequence of a peptide or protein serving as the active ingredient. Said vectors can be conventional viral and non-viral vectors, as are known to the expert. When using viral vectors use can be made of retroviruses, adenoviruses or adeno-associated viruses. In the case of non-viral vectors it is known that no viral DNA participates, so that here fundamentally a "bare" DNA can be introduced into a cell. However, preferably such nucleic acid molecules are packed in so-called liposomes or lipoplexes and are introduced in this form into the organism and cell. The use of non-viral vectors or lipoplexes is fundamentally preferred, because

viral vectors have certain disadvantages known to the expert. As a result of the above-described use possibilities of the invention, it is here frequently possible to operate without using viral vectors, because the effectiveness of the active ingredients used is very high and it is correspondingly possible to operate with low concentrations.

In the invention the active ingredient used is preferably employed in a therapeutically active quantity. In the conventional manner this can be matched to the subject undergoing treatment and inter alia use can be made of known pharmaceutical additives. According to a further development the active ingredient used and correspondingly also the process according to the invention can be provided for local application. This makes it possible to avoid possible disadvantages of a systemic application. The target location of the process according to the invention, namely the inner ear, is particularly suitable for local application. Thus, in the present case the active ingredient can be introduced into the so-called perilymphatic space of the inner ear of the mammal, particularly human. This is a small liquid space with a very slow exchange rate, which is accessible to therapeutic intervention from the middle ear, e.g. via the membrane of the circular window. This perilymphatic space has a volume of only approximately 20 microlitres and is also in direct contact with the cells of the corti-organ. This ensures a direct action of the active ingredient on the sensory epithelium with its hair cells and supporting cells.

The invention also relates to the actual active ingredient, whose use is described in detail in the above-described process. Reference is made to the content and wording of claims 22 to 27. This active ingredient is intended for the regeneration of the sensory cells of the inner ear, particularly the hair sensory cells of the inner ear and is able to at least partly inhibit or eliminate the inhibiting action of a so-called cell cycle inhibitor present in the inner ear. The cell cycle inhibitor is preferably a cyclin-dependent kinase inhibitor, particularly the cyclin-dependent kinase inhibitor $p27^{Kip1}$. Reference is made to the statements hereinbefore concerning the specific, preferred characteristics of the active ingredient. As stated, it can be at least one peptide/protein or at least one nucleic acid molecule, the latter preferably being an antisense-DNA or antisense-RNA or preferably codes for a corresponding peptide/protein usable as the active ingredient. The nucleic acid molecule can be a DNA molecule, a cDNA molecule or a RNA molecule. In particular, the nucleic acid molecule is introduced with the aid of a suitable vector or vehicle into the organism or cell and these can be the described viral or non-viral vectors or nucleic acid molecules packed in liposomes/lipoplexes.

The invention finally relates to a pharmaceutical composition or medicament, which contains at least one active ingredient able to inhibit or eliminate the action of a cell cycle inhibitor present in the inner ear, in an active quantity, as well as conventionally a pharmaceutically acceptable carrier or support. With respect to the active ingredient contained in the composition or medicament express reference is made to the statements hereinbefore and the content of claims 28 and 29.

The described and further features of the invention can be gathered from the following description of a preferred embodiment in conjunction with the subclaims, the example and the drawing. The individual features can be implemented individually or in the form of subcombinations.

Fig. 1 is an electron micrograph of a cell in nuclear division in the sensory epithelium of the corti-organ of a so-called $p27^{Kip1}$ knockout mouse.

Example

For the test use was made of a so-called $p27^{Kip1}$ knockout mouse ($p27^{-/-}$), a mouse lacking the gene for expressing the protein $p27^{Kip1}$. Thus, in such a mouse $p27^{Kip1}$ cannot evolve per se its cell cycle-inhibiting action.

The corti-organ is removed from such a $p27^{Kip1}$ knockout mouse on the seventh day after birth (postnatal day 7) and is prepared in the usual way for electron microscopic examination making it possible to see the sensory epithelium of the corti-organ.

The result of the electron microscopic examination is shown in fig. 1. This electronic micrograph shows that a cell in nuclear division (mitosis), i.e. a mitotic cell is located between two left-hand, upper or right-hand, lower, inner hair cells, whereof the black bordered nuclei are at the left-hand top (complete) and right-hand bottom (partial). Mitosis is clearly visible on the condensed chromatin, the dissolved nuclear membrane and the basal body. the inner hair cell top left and the basal body are given English-language captions in the drawing to facilitate understanding.

Fig. 1 clearly shows that the lack of the cell cycle inhibitor $p27^{Kip1}$ leads to the possibility of a cell division of supporting cells located there in the sensory epithelium of the corti-organ of the mouse. Mention is also made of the fact that in the case of the cell division shown in fig. 1 it is not a single phenomenon within the sensory epithelium of the corti-organ, but instead a large number of the cells there undergo a cell division and therefore pass through the cell cycle. The phenomenon shown in fig. 1

enables the conclusion to be drawn that not only a cell division, but also following a cell division, which represents the decisive step in the hair cell regeneration process, there is also a differentiation or maturation to hair sensory cells and finally a functional recovery of the auditory function of the sensory organ. Thus, a regeneration of the sensory cells is possible. This conclusion is supported by the fact that in the case of the knockout mouse there is not a single mitosis, but instead such knockout mice have more hair cells than normal mice, in which the protein $p27^{Kip1}$ is expressed. Thus, the mitosis of the supporting cells also results in matured sensory cells. The correctness of this conclusion is confirmed by the following results. In the case of heterozygous knockout mice the regeneration of hair cells was proved in that in the second week of living of the animals when they evolve the auditory function, the hair cells were destroyed by the systemic administration of amikacin. After a further two weeks without any injection the animals were killed and their cochlea examined. This revealed regenerated hair cells in the cochlea, which are marked or labelled by a proliferation marker or label (bromodesoxyuridine - BrdU) e.g. administered with the amikacin.

Thus, not only in knockout mice where the gene for $p27^{Kip1}$ was missing from the outset, but also by inhibiting or eliminating the $p27^{Kip1}$ expressed in the normal organism, e.g. with the aid of a peptide interacting with $p27^{Kip1}$ or one of its physiological partners, with the aid of the nucleic acid sequence coding for this peptide or with the aid of an antisense-DNA/antisense-RNA it is possible to bring about a regeneration of the sensory cells. This can also take place by an only partial elimination of the function of $p27^{Kip1}$, because in the case of heterozygous mice a gene dose-dependent effect is observed.

PROCESS FOR THE TREATMENT OF DISEASES OR DISORDERS OF THE INNER EAR

CLAIMS

1. Process for the treatment of diseases or disorders of the inner ear linked with damage or destruction of the sensory cells of the inner ear, characterized in that for the regeneration of the sensory cells of the inner ear the inhibiting action of at least one cell cycle inhibitor present in the inner ear is at least partly inhibited or eliminated by an active ingredient.

2. Use of an active ingredient able to inhibit or eliminate the action of a cell cycle inhibitor present in the inner ear, for the treatment of diseases or disorders of the inner ear linked with damage or destruction of the sensory cells of the inner ear.

3. Use of an active ingredient able to inhibit or eliminate the action of a cell cycle inhibitor present in the inner ear, for the preparation of a pharmaceutical composition or a medicament for the treatment of diseases or disorders of the inner ear linked with damage or destruction of the sensory cells of the inner ear.

4. Process or use according to one of the preceding claims, characterized in that the regeneration of the sensory cells of the inner ear takes place by stimulating proliferation of the supporting cells of the inner ear.

5. Process or use according to one of the preceding claims, characterized in that the sensory cells of the inner ear are hair sensory cells.

6. Process or use according to one of the preceding claims, characterized in that the cell cycle inhibitor is a cyclin-dependent kinase inhibitor.

7. Process or use according to claim 6, characterized in that the cyclin-dependent kinase inhibitor is the cyclin-dependent kinase inhibitor $p27^{Kip1}$.

8. Process or use according to one of the preceding claims, characterized in that the disease or disorder of the inner ear is a perceptive deafness.

9. Process or use according to one of the preceding claims, characterized in that the active ingredient is at least one peptide or at least one protein.

10. Process or use according to one of the preceding claims, characterized in that the active ingredient is at least one nucleic acid molecule, particularly recombined nucleic acid molecule.

11. Process or use according to claim 10, characterized in that the nucleic acid molecule codes for a peptide or a protein according to claim 9.

12. Process or use according to claim 10 or 11, characterized in that the nucleic acid molecule is a DNA molecule.

13. Process or use according to claim 12, characterized in that the nucleic acid molecule is a cDNA molecule.

14. Process or use according to claim 10 or 11, characterized in that the nucleic acid molecule is a RNA molecule.

15. Process or use according to one of the preceding claims, characterized in that the active ingredient is in the form of a vector and preferably the vector carries a nucleic acid molecule according to one of the claims 10 to 14.

16. Process or use according to claim 15, characterized in that the vector is a viral vector.

17. Process or use according to claim 16, characterized in that the virus is a retrovirus, an adenovirus or an adeno-associated virus.

18. Process or use according to claim 15, characterized in that the vector is a non-viral vector.

19. Process or use according to one of the claims 10 to 14, characterized in that it is a nucleic acid molecule packed in a liposome or a lipoplex.

20. Process or use according to one of the preceding claims, characterized in that the active ingredient is used in a therapeutically active quantity.

21. Process or use according to one of the preceding claims, characterized in that the active ingredient is intended for local application.

22. Active ingredient for regenerating the sensory cells of the inner ear, particularly the hair sensory cells of the inner ear, characterized in that it is in a position to at least partly inhibit or eliminate the action of a cell cycle inhibitor present in the inner ear.

23. Active ingredient according to claim 22, characterized in that the cell cycle inhibitor is a cyclin-dependent kinase inhibitor, preferably the cyclin-dependent kinase inhibitor $p27^{Kip1}$.

24. Active ingredient according to claim 22 or 23, characterized in that it is at least one peptide or at least one protein.

25. Active ingredient according to claim 22 or 23, characterized in that it is at least one nucleic acid molecule, preferably a recombined nucleic acid molecule.

26. Active ingredient according to claim 25, characterized in that the nucleic acid molecule is a DNA molecule, cDNA molecule or RNA molecule.

27. Active ingredient according to one of the claims 22 to 26, characterized in that the active ingredient is in the form of a vector or vehicle, which is preferably characterized by at least one of the features of claims 15 to 19.

28. Pharmaceutical composition or medicament, characterized in that it contains at least one active ingredient able to inhibit or eliminate the action of a cell cycle inhibitor present in the inner ear in an active quantity and a pharmaceutically acceptable carrier.

29. Pharmaceutical composition or medicament according to claim 28, characterized in that the active ingredient is an active ingredient according to one of the claims 23 to 27.

PROCESS FOR THE TREATMENT OF DISEASES OR DISORDERS OF THE INNER EAR

ABSTRACT

In a process for the treatment of diseases or disorders of the inner ear linked with damage or destruction of sensory cells of the inner ear, for regenerating the sensory cells use is made of at least one active ingredient, which at least partly inhibits or eliminates the inhibiting action of at least one cell cycle inhibitor present in the inner ear. In this process the sensory cells of the inner ear are preferably regenerated by stimulating the proliferation of supporting cells. The sensory cells of the inner ear are so-called hair sensory cells. As cell cycle inhibitors use can be made of cyclin-dependent kinase inhibitors such as in particular the cyclin-dependent kinase inhibitor $p27^{Kip1}$.

DECLARATION

I, JOHN ALFRED RICHES, Fellow of the Institute of Linguists, of Oak Farm, Catfield, Great Yarmouth, Norfolk, England, do hereby declare that I am conversant with the English and German languages and am a competent translator thereof. I declare further that the following is a true and correct translation made by me of patent application PCT/EP99/01153 (amended pages only) in the German language attached hereto.

Signed this 15th day of August, 2000.

DECLARATION

I, JOHN ALFRED RICHES, Fellow of the Institute of Linguists, of Oak Farm, Catfield, Great Yarmouth, Norfolk, England, do hereby declare that I am conversant with the English and German languages and am a competent translator thereof. I declare further that to the best of my knowledge and belief the following is a true and correct translation made by me of internation patent application PCT/EP99/01153 (amended pages only).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Signed this fifteenth day of August, 2000.

CLAIMS

1. Process for the treatment of diseases or disorders of the inner ear linked with damage or destruction of the sensory cells of the inner ear, characterized in that for the regeneration of the sensory cells of the inner ear the inhibiting action of at least one cell cycle inhibitor present in the inner ear is at least partly inhibited or eliminated by an active ingredient.

2. Use of an active ingredient able to inhibit or eliminate the action of a cell cycle inhibitor present in the inner ear, for the treatment of diseases or disorders of the inner ear linked with damage or destruction of the sensory cells of the inner ear.

3. Use of an active ingredient able to inhibit or eliminate the action of a cell cycle inhibitor present in the inner ear, for the preparation of a pharmaceutical composition or a medicament for the treatment of diseases or disorders of the inner ear linked with damage or destruction of the sensory cells of the inner ear.

4. Process or use according to one of the preceding claims, characterized in that the regeneration of the sensory cells of the inner ear takes place by stimulating proliferation of the supporting cells of the inner ear.

5. Process or use according to one of the preceding claims, characterized in that the sensory cells of the inner ear are hair sensory cells.

6. Process or use according to one of the preceding claims, characterized in that the cell cycle inhibitor is a cyclin-dependent kinase inhibitor.

7. Process or use according to claim 6, characterized in that the cyclin-dependent kinase inhibitor is the cyclin-dependent kinase inhibitor $p27^{Kip1}$.

8. Process or use according to one of the preceding claims, characterized in that the disease or disorder of the inner ear is a perceptive deafness.

9. Process or use according to one of the preceding claims, characterized in that the active ingredient is at least one peptide or at least one protein.

10. Process or use according to one of the preceding claims, characterized in that the active ingredient is at least one nucleic acid molecule, particularly recombined nucleic acid molecule.

Amended sheet

11. Process or use according to claim 10, characterized in that the nucleic acid molecule codes for a peptide or a protein according to claim 9.

12. Process or use according to claim 10 or 11, characterized in that the nucleic acid molecule is a DNA molecule.

13. Process or use according to claim 12, characterized in that the nucleic acid molecule is a cDNA molecule.

14. Process or use according to claim 10 or 11, characterized in that the nucleic acid molecule is a RNA molecule.

15. Process for the treatment of diseases or disorders of the inner ear linked with damage or destruction of the sensory cells of the inner ear, characterized in that for regenerating the sensory cells of the inner ear the inhibiting action of a cyclin-dependent kinase inhibitor present in the inner ear is at least partly inhibited or eliminated by an active ingredient.

16. Process according to claim 15, characterized in that the cyclin-dependent kinase inhibitor is the cyclin-dependent kinase inhibitor $p27^{Kip1}$.

17. Process according to claim 15 or 16, characterized in that the active ingredient is at least one nucleic acid molecule, particularly recombined nucleic acid molecule.

18. Process according to claim 17, characterized in that the nucleic acid molecule is a RNA molecule.

19. Process according to claim 17 or 18, characterized in that the nucleic acid molecule is an antisense sequence.

20. Process or use according to one of the preceding claims, characterized in that the active ingredient is in the form of a vector and the vector preferably carries a nucleic acid molecule according to one of the claims 10 to 14.

21. Process or use according to claim 20, characterized in that the vector is a viral vector.

22. Process or use according to claim 21, characterized in that the virus is a retrovirus, an adenovirus or an adeno-associated virus.

23. Process or use according to claim 20, characterized in that the vector is a non-viral vector.

24. Process or use according to one of the claims 10 to 14, characterized in that it is a nucleic acid molecule packed in a liposome or a lipoplex.

25. Process or use according to one of the preceding claims, characterized in that the active ingredient is used in a therapeutically active quantity.

26. Process or use according to one of the preceding claims, characterized in that the active ingredient is intended for local application.

27. Pharmaceutical composition or medicament, characterized in that it contains at least one active ingredient able to inhibit or eliminate the action of a cell cycle inhibitor, particularly a cyclin-dependent kinase inhibitor present in the inner ear in an active quantity and a pharmaceutically acceptable carrier.

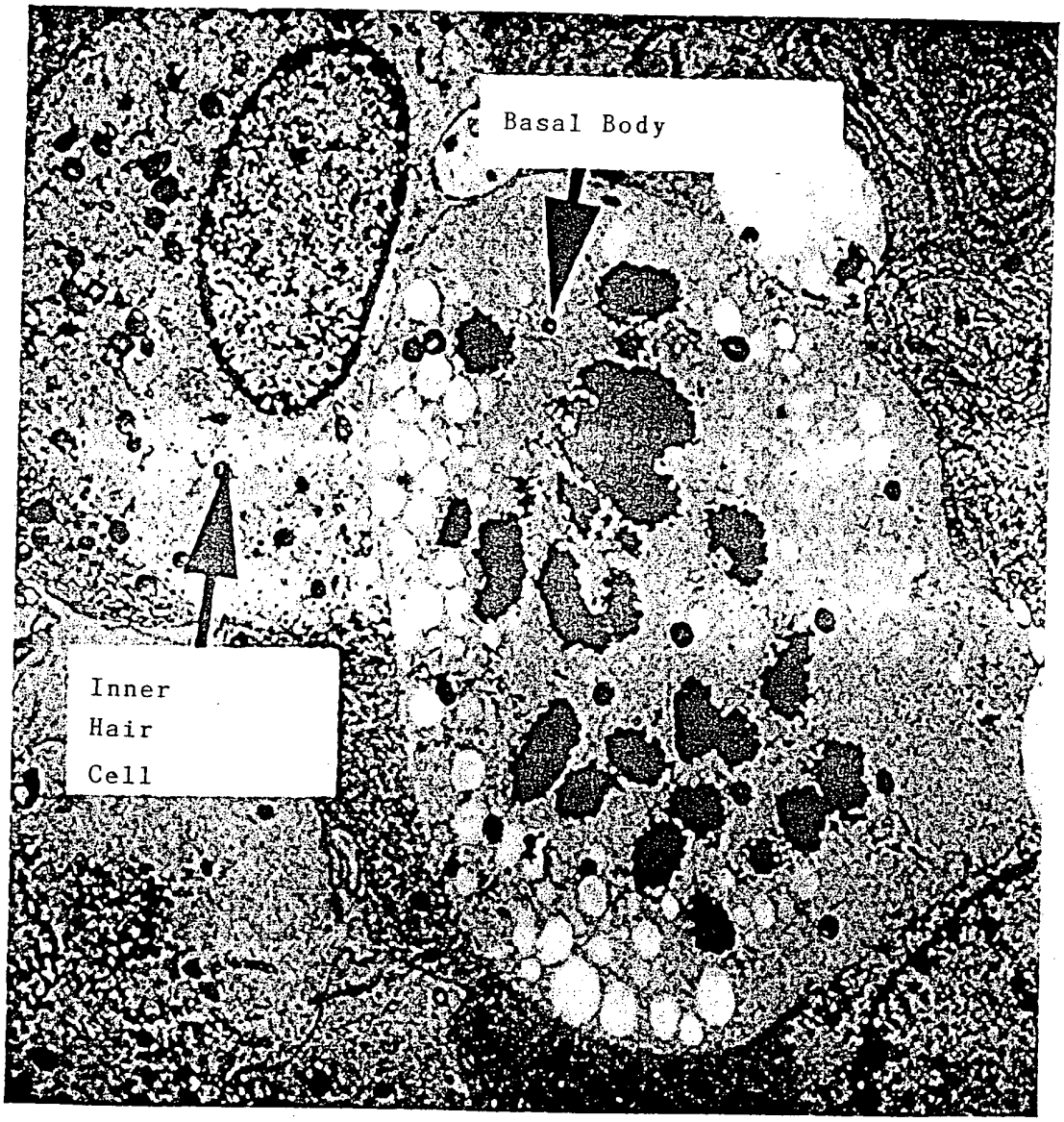

Fig. 1